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(19)(CA) **CANADIAN PATENT** (12)

(54) Antibiotic Polypeptide, Process for Preparing It and the Use Thereof

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Antibiotic polypeptide

The invention relates to an antibiotic polypeptide, to a process for preparing it by means of a new strain of Staphylococcus epidermidis resistant thereto, to the new resistant strain itself, to antibiotic preparation forms containing the active substance, and to the use of the active substance for combating infectious diseases.

It is known from EP-A-0,027,710 published on April 29, 1981 that a particular strain of Staphylococcus epidermidis, namely Staphylococcus epidermidis NCIB 11536 (deposited at the National Collection of Industrial Bacteria, Aberdeen), produces a low-molecular weight antibiotic polypeptide with a broad activity towards gram-positive pathogens, which lyses bacterial cells.

It is also known that the strain Staphylococcus epidermidis MF 205 [S.M. Taylor et al., Int. J. Mass Spectrom, and Ion Physics 48, 161-164 (1983)] likewise produces an oligopeptide effective against gram-positive bacteria. It is not clear from this publication whether the latter strain is identical to the above-mentioned strain NCIB 11536.

It has now been found that a resistant mutant of Staphylococcus epidermidis, which was deposited on 26.10.1984 at the "German Collection of Microorganisms" under the Number DSM 3095, and which is related to the above-mentioned strain NCIB 11536, produces, after a modified preparation and work-up procedure, a similar polypeptide, hereinafter referred to as epidermin, which has an antibiotic effect, chiefly towards gram-positive bacteria. When epidermin is compared with the products from Staphylococcus epidermidis NCIB 11536 and MF 205 it shows, inter alia, considerable differences in amino acid composition.

Comparison of the constituents:

	Epidermin	Products from MF 205	Staph.epidermidis NCIB 11536
Asn	1	-	-
Asx	-	2	1
Glx	-	3	1
Pro	1	1	1
Gly	2	2	2
Ala	2	2	1
Ile	2	2	1
Phe	2	2	1
Lys	2	2	1
Lan	2	-	-
β-Me-Lan	1	-	-
Dhb	1	-	-
Tyr	1	1	
S-(2-Aminovinyl)-D-cysteine	1	-	-
X	-	1-2	-

Dhb corresponds to α,β-dehydroaminobutyric acid, Asx and Glx indicate Asn/Asp and Gln/Glu, respectively.

The following description of the invention will refer to the appended drawings which represent:

Figure 1: Amino acid chromatogram of the acid total hydrolysate of epidermin; ninhydrin coloration, λ = 570 nm.

Figure 2: Gas chromatogram of the n-propylesters of N-pentafluoropropionylamino derivatives of the acid total hydrolysate of epidermin on Chirasil-Val. Temperature programme 3 minutes at 85°C, isothermic, then 4°C per minute up to 200°C; carrier gas H_2 (0.92 bar).

Figure 3: Ultra-violet spectrum of epidermin in water, pH 3 (C=0.15 mg/ml).

Figure 4: Infra-red spectrum of epidermin in a potassium bromide tablet.

Figure 5: ^{1}H nuclear magnetic resonance spectrum of epidermin (20 mg/0.5 ml D_7-dimethylformamide, 400.16 MHz).

Figure 6: ^{13}C nuclear magnetic resonance spectrum of epidermin (40 mg/0.5 ml $^{12}C,^{2}H$-dimethylformamide, 100.6 MHz, 45360 pulses).

Figure 7: HPLC chromatogram of epidermin on µ Bondapak* C_{18} (300x3.9 mm). Mobile phase: A=acetonitrile/0.01 M KH_2PO_4 (10/90), B=acetonitrile/0.01 M KH_2PO_4 (70/30); linear gradient of 10% B to 100% B in 30 minutes, flow rate 2 ml/min.

Figure 8: ^{13}C nuclear magnetic resonance spectrum of the fragment P2 (12 mg/0.5 ml $^{12}C,^{2}H$-dimethylformamide, 100.6 MHz, 38300 pulses).

Figure 9: Isolation of epidermin.

Figure 10: Comparison of the media in terms of the number of living bacteria (continuous lines) and activity against Micrococcus luteus (broken lines).

▲ Brain Heart Infusion medium

○ 3% meat extract, 2% malt extract, 0.25% $CaCO_3$

● 3% meat extract, 2% malt extract, 0.37% $Ca(OH)_2$

Figure 11: Course of fermentation on a 10 litre scale.

● pH curve

▲ number of living bacteria

○ activity against Streptococcus pyogenes ATCC 8668

Figure 12: Comparison of strain NCIB 11536 known from the literature with resistant clone DSM 3095 by means of the number of living bacteria (continuous lines) and activity against Micrococcus

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luteus ATCC 9341 (broken lines).

o strain known from the literature

▲ resistant clone

Figure 13: Addition of epidermin at various growth phases in Staphylococcus epidermidis NCIB 11536.

Curve 1: normal growth pattern

Curve 2: addition of 10 µg/ml of epidermin at the start of the logarithmic phase (A) results in lysis.

Curve 3: addition of 10 µg/ml of epidermin in the middle of the logarithmic phase (B) also results in lysis.

Curve 4: addition of 2.5 µg/ml of epidermin in the middle of the logarithmic phase (B) leads to retardation of growth.

Figure 14: Addition of epidermin at various growth phases in the resultant clone DSM 3095.

Curve 1: normal growth pattern, identical to the strain NCIB 11536.

Curve 2: addition of 120 µg/ml of epidermin at the beginning of the logarithmic phase (A) produces only a slight retardation of growth.

Curve 3: addition of 360 µg/ml of epidermin in the middle of the logarithmic phase (B) again results only in a retardation of growth.

Figure 15: Gas chromatogram of the n-propylesters of N-pentafluoropropionylamino derivatives of the total hydrolysate of H1 on Chirasil-Val.

Temperature programme 3 minutes at 80°C, isothermic, then 4°C per minute up to 200°C; carrier gas H_2.

After total hydrolysis of epidermin using 6N hydrochloric acid (18 hours at 110°C) quantitative amino acid analysis by ion exchange chromatography (standard programme using the Biotronik* LC 6000 E apparatus) gave the following α-amino acid composition:

Asp (1.00), Pro (0.95), Gly (2.09), Ala (2.03), Ile (2.03), Tyr (0.30), Phe (2.02) and Lys (2.00) (cf. Fig. 1). By adding thioglycolic acid the tyrosine content of the total hydrolysate was increased to the substantially stoichiometric value of 0.93.

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The configuration of the protein amino acids was determined by gas chromatography of the n-propyl esters of N-pentafluoropropionyl derivatives of the amino acids obtained from the total hydrolysate after derivatisation. Separation was effected on the chiral stationary phase L-valine-tert.butylamide-polysiloxane (Chirasil-Val). By comparison with a test mixture of amino acids of known configuration the constituents listed above could be categorised as having the L configuration (cf. Fig. 2).

In addition to these protein amino acids the following non-protein amino acids are present: lanthionine (2), β-methyllanthionine (1) and α,β-dehydroaminobutyric acid (1); lanthionine has the meso configuration whilst β-methyllanthionine has the 2S,3S,6R configuration. In addition, epidermin contains an S-(2-aminovinyl)-D-cysteine component.

Epidermin is characterised by the following data:

1. Nature: colourless powder.
2. Solubility: very readily soluble in mixtures of water/glacial acetic acid or methanol/glacial acetic acid, soluble in lower alcohols, insoluble in chloroform, acetone, diethyl ether, petroleum ether.
3. Colour reaction on silica gel plates: ninhydrin, chlorine/TDM [TDM=4,4'-bis-(dimethylamino)-diphenylmethane], orcin/sulphuric acid, anisaldehyde/sulphuric acid. Non-destructive detection in UV light at 254 nm and by spraying with water.
4. Thin layer chromatography on ready-made silica gel plates 60 F_{254} (Merck):
   System A: chloroform/methanol/17% ammonia (2/2/1) $R_F = 0.73$;
   System B: chloroform/methanol/

17% ammonia (70/35/10) R_F = 0.30;
System C: n-butanol/glacial acetic acid/
water (4/1/1) R_F = 0.05.

5. HPLC: see Fig. 7.
6. Stability: stable from pH 2 to pH 7; a sharp drop in activity occurs at higher pH values.
7. Molecular mass: in the region of 2160. Depending on the method of isolation used, epidermin may be isolated as a salt containing anion such as, for example, chloride, acetate or phosphate anions.
8. Ultra-violet absorption spectrum: in aqueous solution, long-wave maximum at 267 nm (Fig. 3).
9. Infra-red absorption spectrum: see Fig. 4.
10. Nuclear magnetic resonance spectra:
^{1}H-NMR spectrum: see Fig. 5.
^{13}C-NMR spectrum: see Fig. 6.

Suitable fragments for sequencing epidermin were obtained by tryptic cleavage. Reaction with trypsin rapidly resulted in a loss of antibiotic activity. Macroscopically, a jelly-like white precipitate was observed upon tryptic cleavage and this precipitate could be removed by centrifugation. The supernatant was lyophilised and subjected to gel chromatography on Sephadex* G 25 (dextran gel made by Pharmacia) in 1% acetic acid.

The chemically uniform fraction (hereinafter referred to as P1), which can be stained with ninhydrin, chlorine/TDM or water, proved to be the N-terminal fragment from the tryptic cleavage of epidermin (see below).

The extremely hydrophobic, jelly-like precipitate consisted of several chemical components which were formed during tryptic cleavage of the C-terminal fragment of the total molecule. A chemically uniform product (hereinafter referred to as P2) was obtained

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by dissolving the precipitate in dimethylformamide, with subsequent gel chromatography on Sephadex* LH-20 (dextran gel produced by hydroxypropylation of Sephadex* G-25, Pharmacia) using dimethylformamide as eluant. P2 could be detected with water or chlorine/TDM, or under UV light. The ninhydrin reaction on P2 proved negative.

Amino acid analysis of the fragment P1 gave the following composition: Pro (1), Lan (1), β-Me-Lan (1), Gly (1), Ala (2), Ile (2), Phe (1), Lys (2). Since isoleucine was determined to be the n-terminal amino acid by dansylation of the total molecule, and since the fragment P2 does not contain isoleucine, the fragment P1 was reasoned to be the N-terminal cleavage product of the total molecule. The C-terminal amino acid of fragment P1 was reasoned to be lysine, from the results of the tryptic cleavage.

In order to clarify the structure of P1 further, the C-terminal amino acid lysine was enzymatically removed by means of carboxypeptidase B (Boehringer Mannheim). The resulting fragment P12 could be isolated in pure form by gel chromatography on Sephadex* G-25 (1% acetic acid). P12 consists of the following amino acids: Pro (1), Lan (1), β-Me-Lan (1), Gly (1), Ala (2), Ile (2), Phe (1), Lys (1).

The sulphur bridges of the thioether amino acids lanthionine and β-methyllanthionine prevent sequence analysis according to the Edman procedure. By reacting P12 with Raney-Ni W2, a sulphur-free dodecapeptide was obtained. meso-Lan was converted into D- and L-alanine and β-methyllanthionine was converted into D-aminobutyric acid and L-alanine. The dodecapeptide sequence was clarified by Edman degradation and FAB spectrometry:

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Ile^{1}-Ala^{2}-Ala^{3}-Lys^{4}-Phe^{5}-Ile^{6}-Ala^{7}-Abu^{8}-
Pro^{9}-Gly^{10}-Ala^{11}-Ala^{12}.

Various investigations for the arrangement of the sulphur bridges in the fragment P12 yielded the following structure:

The C-terminal fragment P2 obtained by tryptic cleavage was able to be characterised as follows: Asn (1), Lan (1), Gly (1), Phe (1), Tyr (1), α-ketobutyric acid and S-(2-aminovinyl)-D-cysteine.

The α-ketobutyric acid originates from the αβ-dehydroaminobutyric acid which directly follows $lysine^{13}$ in the sequence of the total molecule. Tryptic cleavage liberates the amino group of the αβ-dehydroaminobutyric acid; since dehydroamino acids with a free amino group are unstable, they are converted, inter alia, into α-keto acids.

The tryptic fragment P2 is blocked at the N-terminus by the α-ketobutyric acid residue (ninhydrin reaction on P2 proves negative). In addition to the amino acids lanthionine, glycine, phenylalanine, tyrosine and aspartic acid, which are identified by total hydrolysis and amino acid analysis, P2 possesses a further component, which is destroyed by acid total hydrolysis. This was able to be characterised by means of its signals in the ^{13}C-nuclear resonance spectra of epidermin (Fig. 6) and of the fragment P2 (Figure 8).

An indication of the chemical nature of this amino acid was obtained by reacting P2 with Raney-Ni

W2, from which two main products were formed. Both products isolated by preparative HPLC exhibited, after total hydrolysis, an additional D-alanine group in the amino acid chromatogram which was obtained from this reaction.

The structure of this component could be clarified by heterogeneous hydrogenation of the native antibiotic. The four reaction products H1, H2, H3 and H4 were purified by semi-preparative HPLC, totally hydrolysed and subjected to gas chromatography on a chiral stationary phase (Fig. 15). Compared with the chromatogram in Fig. 2, an additional peak occurred in H1 and H3 which was identified by its mass spectrum as being S-(2-aminoethyl)-D-cysteine. In epidermin, this amino acid is in fact present as the acid-unstable component S-(2-aminovinyl)-D-cysteine.

The two peptides P21 and P22 formed from the C-terminal fragment P2 by treatment with Raney nickel W2 were able to be characterised as follows by amino acid analysis and gas chromatography on Chirasil-Val:

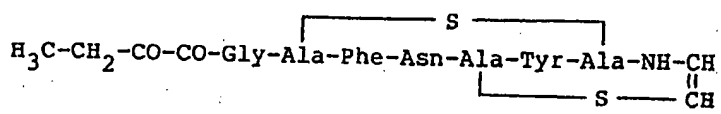
Reaction products	P 21	P 22
Amino acid analysis and gas chromatography on Chirasil-Val	2 D-Ala	1 D-Ala
	1 L-Ala	meso-Lan
	1 L-Phe	1 L-Phe
	1 L-Tyr	1 L-Tyr
	1 L-Asp	1 L-Asp
	1 Gly	1 Gly

In addition, both products were blocked at the C-terminus by ethylamine. Upon desulphurisation with simultaneous hydrogenation S-(2-aminovinyl)-D-cysteine decomposed to form D-alanine and ethylamine.

Sequencing of the bridge-free heptapeptide P21 (partial hydrolysis; 0.1 N hydrochloric acid, 93°C, 16 hours) yielded the following structure:

$H_3C-CH_2-CO-CO-Gly-D-Ala-L-Phe-L-Asn-D-Ala-L-Tyr-L-Ala-NHEt$

The arrangement of the meso-lanthionine bridge present in P22 could be reconciled, by partial hydrolysis and various chemical investigations on enzymatically-derived fragments of P21 and P22, only with the following structure for the fragment P2 derived from tryptic cleavage:

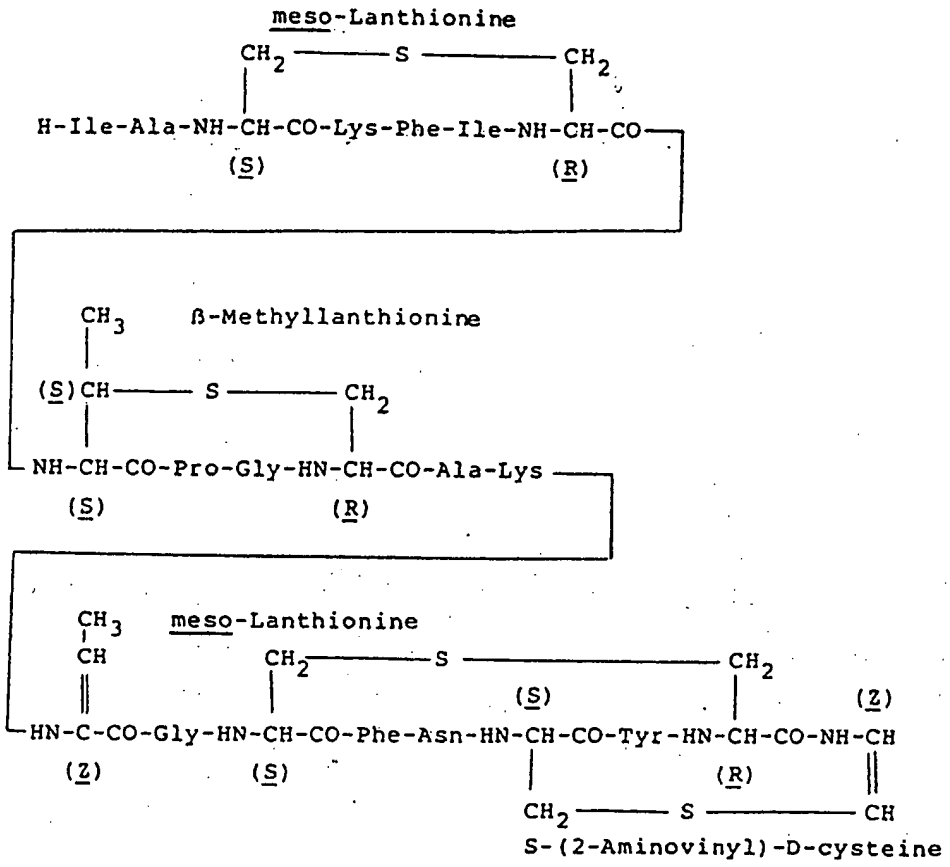


Formal substitution of the 2-ketobutyric acid, which blocks the P2 fragment at the N-terminal, by the amino acid αβ-dehydroaminobutyric acid, which is present in the native antibiotic, and an associated fragment condensation of the tryptic peptides P1 and P2, led directly to a self-consistent amino acid sequence for the ribosomally-synthesised, heterodet tetracyclic peptide antibiotic epidermin.

Thus, according to one feature of the present invention, there is provided an antibiotically-active polypeptide possessing the following amino acid composition:

Asn (1), Pro (1), Gly (2), Ala (2), Ile (2), Phe (2), Lys (2), Lan (2), β-Me-Lan (1), Dhb (1), Tyr (1), S-(2-aminovinyl)-D-cysteine (1)

and having the following primary structure:



wherein the halves of the individual thioether amino acids closer to the N-terminus are of D configuration (which corresponds to the S configuration in the R,S nomenclature).

The polypeptide according to the invention may, for example, be prepared by the following process, which process constitutes a further feature of the present invention:

The producer, Staphylococcus epidermidis DSM 3095, is cultured aerobically at 34-37°C in

a complex nutrient solution consisting of 2 to 4% of a nitrogen source such as, for example, meat extract, 1 to 3% of a carbon source such as, for example, a sugar or a sugar alcohol (e.g. malt extract) and 0.25 to 1% of a carbonate and/or 0.25 to 0.5% of a hydroxide of an alkaline earth metal [e.g. $CaCO_3$ and $Ca(OH)_2$ respectively]. (The percentages quoted herein are expressed throughout as percent by weight, unless otherwise specified).

The maximum antibiotic activity is achieved after 18-23 hours.

Attempts to concentrate the antibiotic were made, for example, on culture filtrate from 10 litre fermenters, and the effectiveness of individual steps was checked by the plate diffusion test. The active component could be concentrated by n-butanol extraction of the culture liquor, which had been freed from extraneous cells and inorganic salts beforehand. Extraction with n-butanol, however, was only possible at the natural end-pH of 8.0 of the culture liquor. Further purification could be achieved by separating off the lipid contaminants by ether precipitation. For this, the n-butanol extract was evaporated, and the residue was dissolved in methanol and stirred into five times the quantity of cold diethyl ether. The activity was left behind completely in the precipitate (Figure 9).

Another particularly suitable method of concentration is adsorption of the centrifuged culture supernatant on Amberlite*XAD-8 or related types of polymeric resin based on acrylate esters (Serva*) or polystyrene. The attachment of epidermin is effected not by simple adsorption but by, for example, the cation-exchanging activity of free acrylic acid groupings in the resin. This is confirmed by the fact that the active component can only be released from the resin by elution with a strongly acidic eluant

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such as, for example, methanol/conc. HCl (99:1). The strongly acidic eluate has to be neutralised with ammonia before being concentrated by evaporation in vacuo. After work-up by adsorption on Amberlite* XAD-8 there is no necessity for subsequent reprecipitation from methanol/ether.

Isolation of the epidermin by adsorption may also be carried out directly from the culture broth during the cultivation of the microorganism.

Stability tests and chromatographic investigations were carried out on the lyophilised n-butanol extract. Incubation of the extract with aqueous solutions of different pH values resulted in a sharp decrease in activity from pH 10 onwards, whereas the antibiotic is stable in the range from pH 2 to 7.

Thin layer chromatography of the evaporated n-butanol extract showed a plurality of compounds which can be stained with various spray reagents. Bioautograms carried out in parallel yielded important indications as to the nature of the new active substance. In acidic and nearly all neutral systems the antibiotic remained on the baseline in thin layer chromatography on silica gel 60*, whereas chromatography with alkaline eluants gave R_F values of between 0.3 and 0.75. The combination of bioautography with thin layer chromatography in alkaline systems showed a correlation with a compound which could be stained with ninhydrin.

The result of these preliminary tests was that the antibiotic is a strongly basic peptide which could not be further purified by column chromatography on silica gel 60 since it could not be eluted from the column with acidic or neutral systems.

Gel chromatography of the isolated material (i.e. of the material isolated from the eluate obtained from the polymeric resin, e.g. from Amberlite* XAD-8, or of the lipid-free n-butanol extract)

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on, for example, Sephadex* LH-20, eluting with, for example, methanol/acetic acid (95:5), separated a large number of small peptides, amino acids and salts in the medium from the antibiotic itself (Fig. 9).

The material thereby obtained was then subjected to multiplicative countercurrent distribution following the procedure developed by Craig, for which it was possible to draw on the experience gained during extraction of the antibiotic from the culture filtrate. In a first, liquid-liquid, distribution system such as, for example, n-butanol/ethyl acetate/0.1 N acetic acid (3:1:3) the active substance remained at the starting point. In a second, neutral, Craig distribution system such as, for example, 2-butanol/0.05 N ammonium acetate (1:1) the active substance moved to a position in the apparatus from which it could conveniently be isolated, e.g. the mid-point of the apparatus.

(Where applicable, the ammonium acetate could be removed by lyophilisation under high vacuum).

After freeze drying, the active substance was obtained in a form which was uniform in all the thin layer systems used.

The purification/isolation procedure according to the invention yields a uniform, pure product, and is thereby superior to the procedure described in EP-A-0,027,710, which comprises freezing/thawing extraction, evaporation of the extracting agents, ultrafiltration, precipitation with ammonium sulphate, ion exchange chromatography and gel filtration on Sephadex* G-50, G-25 or G-15 or on Biogel* P2. The two procedures thus differ fundamentally.

A notable difference between the process according to the invention and that described in EP-A-0,027,710 resides in the choice of the complex nutrient solution. Whereas the known nutrient

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solution Brain Heart Infusion (37 g BHI/l) gave only a very small yield of antibiotic, a nutrient solution according to the invention consisting of 2 to 4% meat extract, 1 to 3% sugar or sugar alcohols (such as, for example, malt extract, maltose, galactose, lactose, mannitol, glucose or glycerol) and 0.25 to 1% calcium carbonate or 0.25 to 0.5% calcium hydroxide, gave very good results. The best production was achieved with a nutrient solution having the following composition: 3% meat extract, 2% malt extract and 0.37% calcium hydroxide. Of all the carbon sources utilised, maltose gave the best production after malt extract. Glucose is preferably used only in conjunction with other carbon sources. A combination of lactose and maltose, or galactose and maltose, also gave good results. All the other conventional carbon sources showed no or very little production.

The addition of all 20 amino acids as a nitrogen source, in concentrations of 2 g/l each, gave the same results as the meat extract. Casamino acid (Difco) is a suitable nitrogen source only if tryptophan (1-2 mM) and one or more vitamins are added. Suitable vitamins include (preferred concentrations being shown in brackets): biotin (0.006 mg/l), nicotinic acid (2.3 mg/l), thiamine (1.0 mg/l), pyridoxine.HCl (12.0 mg/l) and calcium pantothenate (1.2 mg/l).

Fermentation is most effectively carried out with good ventilation at temperatures of between 34 and 37°C. The optimum production pattern is obtained if the pH of the culture medium is adjusted to a value of between 6.0 and 7.0 before fermentation. In the absence of carbonates or hydroxides of divalent cations such as, for example, calcium carbonate or calcium hydroxide, no production takes place. After the addition of calcium carbonate, for example, the pH value shows a characteristic pattern, falling

into the acid range, in which case no production occurs. When the pH value subsequently rises into the slightly alkaline range production commences. Instead of calcium carbonate it is also possible to use magnesium carbonate, whilst calcium hydroxide gives better results than calcium carbonate. With 50 mM calcium hydroxide, production can be increased compared with that obtained with 25 mM calcium carbonate. When the carbon sources (sugars) are utilised by the strain, organic acids are formed which are complexed by divalent cations and at the same time the medium is buffered.

Figure 10 shows the results in terms of the curve representing the number of living bacteria, and the production of antibiotic expressed in mm of inhibitory area against Micrococcus luteus ATCC 9341, of the media according to the invention compared with Brain Heart Infusion Medium (Difco) according to EP-A-0,027,710.

The following increases in activity at the moment of maximum production were obtained in the plate diffusion test using a calibrated line. The activity in Brain Heart Infusion nutrient solution was equated with 100%.

a) Brain Heart Infusion Agar	100%
b) 3% meat extract, 2% malt extract, 25 mM calcium carbonate	200%
c) 3% meat extract, 2% malt extract, 50 mM calcium hydroxide	320%

These data do not constitute absolute production values. However, a comparison of the values achieved in the plate diffusion test clearly shows that a significant increase in yields is achieved by using the procedure according to the invention compared with the known procedure.

The strain used to produce epidermin, which constitutes a further feature of the present invention, is characterised as follows according to Schleifer & Kloos [Int. J. Syst. Bact. 25, 50-61 (1975)]:

Gram coloration	: positive
Cell size	: 0.5-0.8 µm in diameter
Colony size	: about 1 mm in diameter
Appearance of colonies	: smooth, glossy, slightly raised in the centre
Colour of colonies	: greyish-white
Cells in culture	: often single cells or groups of two, seldom larger clumps
Haemolysis	: cultures spread on blood slides showed strong haemolysis
Anaerobic growth	: the strain grows even under anaerobic conditions
Lysozyme sensitivity	: the cells are resistant to lysozyme in quantities of up to 2 mg/ml
Lysostaphin sensitivity	: the cells are sensitive to lysostaphin even in quantities of 20 µg/ml
Epidermin resistance	: demonstrated up to 1 mg/ml
Other resistances	: in liquid culture the strain shows a marked resistance to Streptomycin (up to 1 mg/ml) and Spectinomycin (0.5 mg/ml)
Increased NaCl content	: the strain still grows well with a sodium chloride content of up to 15% by weight.

Colonies or spread-out cultures are very adhesive.

The utilisation of various carbon sources with acid formation and other enzyme reactions was determined using the Api-Staph System (Biomerieux, Nürtingen). This system is based on a combination of 20 biochemical reactions which can be traced back to the classification of Kloos & Schleifer [J. Clin. Microbiol. 1, 82-88 (1975)].

Table 1: Utilisation of carbon sources and other enzyme reactions

Carbon source or enzyme	Producer	Staph. epid. (Kloos & Schleifer)	Staph.epid. (Api - Staph)
Control	-	-	-
D-Glucose	+	n.d.	+
D-Fructose	+	+	+
D-Mannose	(+)	(+)	+
Maltose	+	+	+
Lactose	(+)	(+)	+
D-Trehalose	-	-	-
D-Mannitol	-	-	-
Xylitol	-	-	-
D-Melibiose	-	n.d.	-
Raffinose	-	n.d.	-
D-Xylose	-	-	-
Sucrose	+	+	+
α-Methylglucoside	-	n.d.	-
N-Acetylglucosamine	-	n.d.	±
Nitrate reduction	+	+	±
Phosphatase	+	+	+
Formation of acetyl-methylcarbinol (Voges-Proskauer reaction)	+	n.d.	+
Arginine dehydrolase	-	n.d.	±
Urease	+	n.d.	+

\+ positive reaction
\- negative reaction
(+) clearly positive reaction does not occur until later
± variable result
n.d. no data

The strain Staphylococcus epidermidis DSM 3095 was inoculated on slanting tubes with a medium having the following composition:

Peptone	10	g
Disodium hydrogen phosphate	2	g
Meat extract	8	g
Glucose	10	g (autoclaved separately)
Common salt	3	g
pH	7.2.	

The inoculum was then incubated overnight at 37°C and subsequently frozen at -20°C. A fresh tube was thawed for each new test mixture since losses of activity were observed on lengthy storage.

A more precise description of the preparation of the antibiotic epidermin will now be given by way of Example:

Fermentation is conveniently carried out in suitable shaking flasks and, in order to prepare larger quantities of active substance, fermenters with a capacity of 200 litres or more may be used.

For flask tests, 500 ml Erlenmeyer flasks with a lateral inlet were used. The flasks were filled with 100 ml of nutrient solution and autoclaved for 20 minutes at 121°C. The inoculant used was 1% of a preliminary culture which was 4 hours old. Incubation was carried out at 37°C in a shaking machine rotating at 140 rpm.

For fermentation on a 10 litre scale, fermenters with a useful capacity of 10 litres (Model MF-14* made by New Brunswick Scientific Co., New Brunswick, USA) were filled with 9.9 l of nutrient solution and autoclaved for 30 minutes at 134°C. After cooling, the nutrient solution was inoculated with 100 ml of a 4-hour-old preliminary culture and fermented at 37°C and 0.6 vvm, at a blade-stirrer

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speed of 240 rpm. The relatively strong foaming could be counteracted by repeatedly adding sterile polyol.

For fermentation on a 25 litre scale, a 25 litre fermenter (Type b 25 made by Braun/Melsungen, with a recirculating system) was filled with 25 l of nutrient solution, with the addition of 3 ml of polyol, and sterilised in situ at 121°C for 30 minutes. The inoculant used was 300 ml of a preliminary culture which was 4 hours old. The fermentation was carried out at 37°C, 0.6 vvm and 1000 rpm.

The course of fermentation on a 10 litre scale in a nutrient solution containing 30 g of meat extract, 20 g of malt extract and 5 g of calcium carbonate per litre is shown in Figure 11. Intensive growth combined with the utilisation of the carbon source provided, with formation of acid, can be detected as the pH value falls. With the start of alkalisation the antibiotic can be detected in the culture filtrate. Production reaches a peak after 12 to 18 hours.

In order to monitor the course of fermentation, samples were taken under sterile conditions at various times during fermentation. The samples were evaluated as follows:

a) pH value:
Measured with a laboratory pH meter (Knick pH mV meter)

b) Course of growth:
Growth could be monitored by observing the increase in the number of living bacteria. To this end, 0.5 ml of culture taken under sterile conditions was diluted in saline and 0.1 ml of this dilution was spread onto slides (medium: peptone 10 g, meat extract 8 g, common salt 3 g, disodium hydrogen phosphate

2 g, glucose 10 g per litre). After 18 hours' incubation at 37°C the individual colonies could be counted.

c) Concentration of antibiotic:
The samples were centrifuged in an Eppendorf centrifuge 3200 for 2 minutes and 20 µl of the supernatant was tested in a plate diffusion test. At the same time a calibration curve was plotted with known concentrations.

After maximum production had been reached the culture liquid was centrifuged off at 1380 rpm by continuous centrifuging (centrifuge: Type LA 71b-4*, Loher & Söhne, Ruhstorf/Rott). For optimum separation of the cells the flow rate had to be kept very low. A first concentration of the active components was achieved using the adsorption on Amberlite*XAD-8 mentioned hereinbefore, as described in the following Example 1:

Example 1

80 litres of culture filtrate was poured over about 8 litres of Amberlite*XAD-8. No activity could be detected in the throughflow. When subsequent washing with water was carried out again no activity was eluted. Subsequently, washing was carried out with 13 litres of methanol. No activity was detected in the washing solvent. Elution was effected using methanol:hydrochloric acid 99:1, and the following fractions were obtained:

Fraction 1: 0.8 l; active
Fraction 2: 4.5 l; main activity
Fraction 3: 2 l; active
Fraction 4: 2 l; no activity
Fraction 5: 1 l; no activity

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The fractions were tested by thin layer chromatography for their epidermin content, then the active fractions were combined and concentrated by evaporation to dryness in a rotary evaporator (81.2 g). The residue was taken up in methanol/acetic acid (95:5) (400 ml), centrifuged to remove the insoluble components and subjected to gel chromatography in batches of 50 ml on Sephadex*LH-20 (column 100x5 cm, eluant methanol:acetic acid 95:5). Positive fractions were combined and concentrated by evaporation (14.8 g).

For multiplicative distribution according to Craig, apparatus made by Labortec (Basle) were used. A first separation was carried out in a 50 ml apparatus using a system of n-butanol/ethyl acetate/0.1 M acetic acid (3:1:3). The sample (5 g) dissolved in the lower phase (100 ml) was subjected to the following separation conditions:

Number of stages:	160
Vibrating movements per stage:	70
Intensity of vibration:	45
Separating time:	20 min.

For final purification the crude product obtained (4.2 g) was dissolved in batches of 2 g in the lower phase of a 2-butanol/0.05 N ammonium acetate (1:1) system (50 ml) and purified in a 10 ml apparatus with 440 elements.

Number of stages:	440
Vibrating movements per stage:	50
Intensity of vibration:	50
Separating time:	10 min.

The elements containing epidermin were combined, concentrated by evaporation, taken up in water and lyophilised several times under high vacuum.

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The antibiotic (2.6 g) proved to be uniform in all the tests carried out.

During the preparation and isolation of the epidermin the plate diffusion test was used for biological characterisation, namely to record the activity spectrum and to monitor production, work-up and concentration. The test was carried out in Petri dishes made by Greiner, Nürtingen, using filter discs 6 mm in diameter (Macherey & Nagel, Düren) as described in Zähner and Maas, Biology of Antibiotics (Springer Verlag, Berlin-Heidelberg-New York, 1972). The filter discs were wetted with 20 µl of the appropriate test liquid, dried on a glass slide at ambient temperature and placed on the test plates. The test plates were incubated at 37°C and the inhibition of growth was evaluated after about 16 hours. The routine test bacteria used were Streptococcus pyogenes ATCC 8668 and, owing to its less problematic handling, Micrococcus luteus ATCC 9341.

Test plates with Streptococcus pyogenes ATCC 8668: Before preparation of the test plates the test bacteria had to be freshly inoculated on blood plates with mucin [medium: 500 ml of agar, pH 7.4; 160 ml of mucin solution (10% by weight); 3.5 ml of glucose solution (50% by weight); and 70 ml of sheep's blood]. After 15 to 18 hours' incubation at 37°C a saline suspension (E_{578} 0.5) was prepared and 1 ml of this suspension was pipetted into 100 ml of nutrient base (medium: peptone 10 g, meat extract 8 g, common salt 3 g, Na_2HPO_4 2 g, glucose 10 g per litre, pH 7.2). The plates carrying 10 ml batches could be stored for several days at 4°C.

Test plates with Micrococcus luteus ATCC 9341: An overnight culture (medium: peptone 10 g, meat extract 8 g, common salt 3 g, Na_2HPO_4 2 g, glucose

10 g per litre, pH 7.2) was diluted to an extinction of 1.0 at 578 nm. 100 ml of agar was inoculated with 0.25 ml of this suspension and 10 ml batches were poured over slides. The test plates could be stored for several days at 4°C.

Test plates for the activity spectrum:

Test plates with bacteria:

α) Aerobic bacteria:

Overnight cultures were grown in the relevant test media and the plates were prepared as described for Micrococcus luteus ATCC 9341.

β) Anaerobic bacteria:

The preliminary cultures for Clostridium pasteurianum ATCC 6013 and Propionibacterium acnes DSM 1897 were grown in test tubes which had been filled up to the cotton wool stopper with nutrient solution in order to displace the oxygen. 100 ml of agar was inoculated with 3 ml of the existing culture and 10 ml batches were poured over the plates. Incubation was effected in an anaerobic pot (BBL-Gas Pak*100, Becton, Dickinson GmbH, Heidelberg) at the optimum temperature.

Test plates with yeast-like fungi:

The cells were grown in the relevant test medium for 18-20 hours in a shaken culture. After counting had been carried out in a Thoma counting chamber the test plates were inoculated to a density of 10^6 organisms per ml of agar.

Test plates with fungi and Streptomycetes:

The test organisms were grown on slanting tubes (medium: yeast extract 4 g, malt extract 10 g, glucose 4 g per litre) at the corresponding temperatures until sporulation occurred. The spores

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were floated off with 3 ml of saline Tween* 80 (1 drop of Tween* 80 to 100 ml of saline) and poured into test plates.

Epidermin has a very good antibacterial activity; it is particularly effective against a whole series of gram-positive bacteria. The antibacterial effect of epidermin was tested by comparison with that of nisin (for nisin cf. inter alia DE-A-2,000,818 or GB-B-1,182,156); for a few important, clinically relevant bacteria, fusidic acid was also included in the comparison. The effectiveness was determined by the plate diffusion test and by determining the minimum inhibitory concentrations.

a) Plate diffusion test:

The plate diffusion test investigated the sensitivity of various microorganisms towards epidermin and nisin. Table 2 shows that both these antibiotics act almost exclusively on gram-positive bacteria. Nisin with an activity of 40,000 units was used.

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Table 2: Sensitivity to epidermin and nisin
Given in mm of inhibited area, concentrations 1 mg/ml and 0.5 mg/ml.

Microorganisms	Culture conditions Temp.	Medium	Epidermin 1	Epidermin 0,5	Nisin 1	Nisin 0,5
Bacteria						
Eubacteriales, gram-positive						
Arthrobacter aurescens	27°C	5	8	Sp	9	8
Arthrobacter crystallopoietes	27°C	5	16	15	16	15
Arthrobacter globiformis	27°C	5	14	12	14	13
Arthrobacter oxydans	27°C	5	12	11	11	10
Arthrobacter pascens	27°C	5	13	12	14	13
Bacillus cereus	37°C	3	8	Sp	-	-
Bacillus pumilus	37°C	4	14	12	12	10
Bacillus subtilis ATCC 6051	37°C	4	12	11	9	8
Bacillus subtilis ATCC 6051	37°C	6	17	16	14	12
Bacillus subtilis ATCC 6633	37°C	4	14	11	9	8
Bacillus subtilis F 24-2	37°C	4	13	11	8	Sp
Bacillus subtilis A 14	37°C	4	12	10	9	Sp
Brevibacterium flavum	37°C	4	11	10	8	7
Clostridium pasteurianum	30°C	7	17	15	14	13
Clostridium sporogenes	37°C	8	10	9	8	Sp
Corynebacterium spec.	27°C	4	15	13	9	8
Corynebacterium insidiosum	27°C	4	19	17	10	8
Corynebacterium rathayi	27°C	4	14	12	10	9
Micrococcus luteus ATCC 381	27°C	5	10	9	-	-
Micrococcus luteus ATCC 9341	27°C	2	21	20	17	16
Propionibacterium acnes	37°C	8	22	19	21	18
Sarcina lutea	37°C	5	15	14	15	14
Staphylococcus aureus Tü 202	37°C	4	13	10	10	9

Staphylococcus aureus DSM 683	37°C	4	11	9	Sp	-
Staphylococcus aureus Pen.res	37°C	4	12	11	9	8
Staphylococcus cohnii	37°C	2	14	12	9	-
Streptococcus pyogenes	37°C	2	22	20	18	17
Eubacteriales, gram-negative:						
Proteus mirabilis	37°C	4	11	9	9	7
Proteus vulgaris	37°C	5	9	8	8	Sp
Actinomycetales:						
Streptomyces glaucescens	27°C	3	10	8	10	9
Streptomyces violaceoruber	37°C	3	9	8	-	-
Streptomyces viridochromogenes	37°C	3	11	10	9	8

(-) indicates that there was no activity;
Sp = traces

b) Minimum inhibitory concentration:

The minimum inhibitory concentration for clinically relevant bacteria given in µg/ml was tested in microtitre plates in the following medium:

5 ml sodium lactate, 5 g Na_2SO_4, 0.5 g KH_2PO_4, 0.1 g $MgCl_2$, 5 g NH_4Cl, 10 g glucose, 50 µg calcium pantothenate, 50 µg thiamine, 0.25 µg folic acid, 50 µg niacin, 25 µg p-aminobenzoic acid, 50 µg pyridoxine hydrochloride and 25 µg riboflavin in 1000 ml of distilled water.

Table 3 shows the minimum inhibitory concentrations

(µg/ml) of epidermin, nisin and fusidic acid in a comparative study. The nisin used here had an activity of 2500 units.

Table 3:
Minimum inhibitory concentration in µg/ml:

	Epidermin	Fusidic acid	Nisin
St. epidermidis WG 99	2	4	128
Sc. pyogenes ATCC 8668	0,125	1	4
Sc. pneumoniae ATCC 6302	2	16	32
Mc. luteus ATCC 15957	0,25	0,5	16
Mc. luteus ATCC 9341	$\leq$ 0,06	0,5	8
Cb. xerosis NCTC 9755	0,125	0,125	16
E. coli ATCC 11775	128	>128	>128
E. coli ATCC 9637	128	>128	>128
Propionib. acnes PC 904	0,06	0,5	32
Propionib. acnes ATCC 25746	0,25	1	64

The media mentioned in Table 2 are described hereinafter. The media were autoclaved for 20 minutes at 121°C after the pH value had been adjusted.

The quantities given all refer to 1 litre of water. In the case of chemically defined media, deionised water was used.

Medium:

(2) Glucose-agar medium:

Peptone	10 g
Meat extract	8 g
NaCl	3 g
Na_2HPO_4	2 g
Glucose	10 g (autoclaved separately)
Agar	20 g
pH 7.2	

(3) Yeast-malt medium:

Yeast extract	4 g
Malt extract	10 g
Glucose	4 g
Agar	20 g
pH 7.3	

(4) Oxoid medium for bacteria:

Meat extract	10 g
Peptone	10 g
NaCl	5 g
Agar	20 g
pH 7.2	

(5) Nutrient broth (Difco) 8 g

Agar 20 g

pH 7.2

(6) Minimal medium for bacteria (Hütter et al., 1966):

D-Glucose	8 g
Diammonium tartrate	4 g
NaCl	5 g
K_2HPO_4	2 g
$MgSO_4.7H_2O$	1 g
$CaCl_2$	0.2 g
$MnSO_4.H_2O$	0.01 g
Ferrioxamine B	0.02 g
Agar	20 g
pH 7.2	

(7) Medium for Clostridium pasteurianum:

Meat extract	3	g
Yeast extract	3	g
Malt extract	3	g
Peptone	20	g
D-Glucose	5	g
Ascorbic acid	0.2	g
Agar	20	g

pH 7.0

(8) Brewer thioglycolate medium for Propionibacterium acnes:

Thioglycolate medium	40.5	g
Agar	20	g

pH 7.2

Epidermin is very well tolerated and when used topically no toxic effects are detected.

A major advantage is the fact that the resistant mutant Staphylococcus epidermidis DSM 3095 is resistant to the epidermin which it produces. The strain NCIB 11536 has no such resistance to the low-molecular weight antibiotic which it produces.

The new clone DSM 3095 was obtained as follows:

Adaptation was carried out in 100 ml Erlenmeyer flasks with a lateral insert and 10 ml of nutrient solution (Brain Heart Infusion). The starting culture was inoculated with 0.5 ml of a culture which was 12 hours old. For the flasks which contained increasing concentrations of epidermin, 0.5 ml of the previous, well-grown culture were used as inoculant. The growth was assessed photometrically at 578 nm. The culture, which was still well-grown at a concentration of 1.0 mg/ml of epidermin in liquid culture, was diluted in saline and spread onto a slide into which 0.5 mg/ml of epidermin had been poured. As a comparison, an undiluted 12-hour-old culture of the comparison strain NCIB 11536, known from the literature, was spread on

a plate. After 24 hours' incubation at 37°C, 94 individual colonies were visible in the adapted culture diluted to 10^{-6}. The comparison strain spread out undiluted on plates containing 0.15 mg/ml did not grow even after 48 hours' incubation. The resistant colonies were selected and spread onto plates containing medium 2 in a grid pattern. After a preliminary test for production by pricking out fragments with a diameter of 5 mm and testing the activity on Micrococcus luteus ATCC 9341, productive clones were tested in a liquid medium.

Comparison of the resistant strain with the strain known from the literature:

a) Minimum inhibitory concentration in the plate diffusion test:

The minimum inhibitory concentrations shown in Table 4 were obtained on test plates containing the two strains.

Table 4: Minimum inhibitory concentrations of epidermin in the plate diffusion test with NCIB 11536 and DSM 3095.

Strain	µg/ml
NCIB 11536	10
DSM 3095	> 1000

b) Growth and production:

The growth and production patterns of the two strains were compared in a nutrient solution [3% meat extract, 2% malt extract, 0.37% $Ca(OH)_2$]. See Figure 12.
Growth pattern: determination of number of living bacteria.

Production pattern: activity against Micrococcus luteus ATCC 9341 in the plate diffusion test. Better production is observed with the resistant strain.

c) Sensitivity to epidermin in various growth phases:

Tests on the inherent inhibition of the producing strain by the antibiotic and a test on the resistance of the selected clones were carried out in a biophotometer (Eppendorf photometer with automatic circulating means).

These tests may, for example, be carried out as follows:

0.5 ml batches of a 12-hour-old culture of Staphylococcus epidermidis NCIB 11536 or the selected resistant strain DMS 3095 were inoculated onto fresh medium.

These cultures were left to grow until an extinction of 0.043 at 578 nm was obtained (dishes with a layer thickness of 1 cm) and were then distributed in batches of 7.5 ml in the biophotometer cuvettes (layer thickness 2 cm).

The density of the cell suspension was adjusted to a transmission of 90% in the biophotometer. Incubation was carried out at 37°C with maximum ventilation. Epidermin was added as an aqueous solution in various growth phases.

In both strains, different epidermin concentrations were added at the beginning and in the middle of the logarithmic phase. The results are shown in Figures 13 and 14.

No lysis was observed in the resistant strain with the concentrations tested hitherto.

The antibiotically-active polypeptide according to the invention, like the culture liquor from

which it is isolated, exerts a bactericidal effect which results in lysis of the cells.

In view of its bactericidal activity and broad spectrum activity against gram-positive bacteria, the polypeptide antibiotic epidermin according to the invention is particularly suitable for the treatment of infections caused by gram-positive bacteria. Epidermin is particularly valuable in combating skin infections such as eczema, impetigo, cellulitis and particularly acne. Its extremely good effectiveness against some important strains of Propionibacterium acnes has been shown hereinbefore in Tables 2 and 3. Clearly, therefore, epidermin, which is normally produced by organisms inhabiting human skin, is more effective at protecting the skin than are other conventional antibiotics.

Thus, according to a yet further feature of the present invention there are provided pharmaceutical compositions containing, as active ingredient, an antibiotically-active polypeptide as hereinbefore defined in association with one or more inert pharmaceutical carriers and/or excipients.

For pharmaceutical administration the said polypeptide may be incorporated into preparations in either liquid or solid form using carriers and excipients conventionally employed in the pharmaceutical art, optionally in combination with further active ingredients. The preparations may, for example, be applied orally, parenterally, enterally or, preferably, topically. Preferred forms include, for example, solutions, emulsions, gels, lotions, ointments, creams or powders.

Advantageously the compositions may be formulated as dosage units, each unit being adapted to supply a fixed dose of active ingredient. The total daily dose may, of course, be varied depending on the subject treated and the complaint concerned.

According to a still further feature of the present invention there is provided a method for the treatment of a patient suffering from, or susceptible to, infections caused by gram-positive bacteria, particularly skin infections, which comprises administering to the said patient an effective amount of an antibiotically active polypeptide according to the invention.

The following non-limiting Examples describe the preparation of certain pharmaceutical compositions:

Example 2

Tincture

100 g of tincture contains:

Epidermin	1.0 g
Ethanol (94.5 % by volume)	56.0 g
1,2-Propylene glycol	40.0 g
Demineralised water	3.0 g

Preparation:

Epidermin is dissolved in a mixture of ethanol/1,2-propylene glycol/water and the solution is then filtered sterile.

Example 3

Lotion

100 g of lotion contains:

Epidermin	1.00 g
1,2-Propylene glycol	7.00 g
Alkyldimethylbenzylammonium chloride (Benzalkon*A(R))	0.15 g
Sorbitan monopalmitate (Span*40(R))	0.40 g
Sorbimacrogol palmitate (Tween*40(R))	1.20 g
Decyl oleate (Cetiol*V(R))	2.40 g
Mixture of cetyl and stearyl alcohols (Lanette*O(R))	1.60 g

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Cetyl palmitate		0.80 g
Demineralised water	ad	100 g

Preparation:

The above quantities of alkyldimethylbenzylammonium chloride, sorbitan monopalmitate, sorbimacrogol palmitate, decyl oleate, cetyl and stearyl alcohols and cetyl palmitate are stirred into 75 ml of water, the filtered solution of epidermin in 1,2-propylene glycol and the remaining water are stirred in, and the tincture is homogenised.

Example 4

Gel

100 g of gel contains:

Epidermin		1.0 g
Polyethylene glycol ether of lauryl alcohol (Brij*35 (R))		1.0 g
1,2-Propylene glycol		5.0 g
Acrylic acid polymer (Carbopol* 934 (R))		1.2 g
Methyl p-hydroxybenzoate		1.6 g
Propyl p-hydroxybenzoate		0.4 g
Perfume		q.s.
Sodium hydroxide solution	ad	pH 6.5
Demineralised water	ad	100 g

Preparation:

The specified quantities of excipient are stirred into 75 ml of water; the epidermin is dissolved in a mixture of 1,2-propylene glycol and the remaining water, and this solution is again stirred in; the finished gel is homogenised once more.

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Legend to the figures:

Figure 1: Amino acid chromatogram of the acid total hydrolysate of epidermin; ninhydrin coloration, λ= 570 nm.

Figure 2: Gas chromatogram of the n-propylesters of N-pentafluoropropionylamino derivatives of the acid total hydrolysate of epidermin on Chirasil-Val. Temperature programme 3 minutes at 85°C, isothermic, then 4°C per minute up to 200°C; carrier gas H_2 (0.92 bar).

Figure 3: Ultra-violet spectrum of epidermin in water, pH 3 (C=0.15 mg/ml).

Figure 4: Infra-red spectrum of epidermin in a potassium bromide tablet.

Figure 5: ^1H nuclear magnetic resonance spectrum of epidermin (20 mg/0.5 ml D_7-dimethylformamide, 400.16 MHz).

Figure 6: ^{13}C nuclear magnetic resonance spectrum of epidermin (40 mg/0.5 ml $^{12}C,^2H$-dimethylformamide, 100.6 MHz, 45360 pulses).

Figure 7: HPLC chromatogram of epidermin on μ Bondapak* C_{18} (300x3.9 mm). Mobile phase: A=acetonitrile/0.01 M KH_2PO_4 (10/90), B=acetonitrile/0.01 M KH_2PO_4 (70/30); linear gradient of 10% B to 100% B in 30 minutes, flow rate 2 ml/min.

Figure 8: ^{13}C nuclear magnetic resonance spectrum of the fragment P2 (12 mg/0.5 ml $^{12}C,^2H$-dimethylformamide, 100.6 MHz, 38300 pulses).

Figure 9: Isolation of epidermin.

Figure 10: Comparison of the media in terms of the number of living bacteria (continuous lines) and activity against Micrococcus luteus (broken lines).

▲ Brain Heart Infusion medium

O 3% meat extract, 2% malt extract, 0.25% $CaCO_3$

● 3% meat extract, 2% malt extract, 0.37% $Ca(OH)_2$

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Figure 11: Course of fermentation on a 10 litre scale.
● pH curve
▲ number of living bacteria
O activity against Streptococcus pyogenes ATCC 8668

Figure 12: Comparison of strain known from the literature with resistant clone DSM 3095 by means of the number of living bacteria (continuous lines) and activity against Micrococcus luteus ATCC 9341 (broken lines).
O strain known from the literature
▲ resistant clone

Figure 13: Addition of epidermin at various growth phases in Staphylococcus epidermidis NCIB 11536.
Curve 1: normal growth pattern
Curve 2: addition of 10 µg/ml of epidermin at the start of the logarithmic phase (A) results in lysis.
Curve 3: addition of 10 µg/ml of epidermin in the middle of the logarithmic phase (B) also results in lysis.
Curve 4: addition of 2.5 µg/ml of epidermin in the middle of the logarithmic phase (B) leads to retardation of growth.

Figure 14: Addition of epidermin at various growth phases in the resultant clone DSM 3095.
Curve 1: normal growth pattern, identical to the strain NCIB 11536.
Curve 2: addition of 120 µg/ml of epidermin at the beginning of the logarithmic phase (A) produces only a slight retardation of growth.
Curve 3: addition of 360 µg/ml of epidermin in the middle of the logarithmic phase (B) again results only in a retardation of growth.

Figure 15: Gas chromatogram of the n-propylesters of N-pentafluoropropionylamino derivatives of the total hydrolysate of H1 on Chirasil-Val.

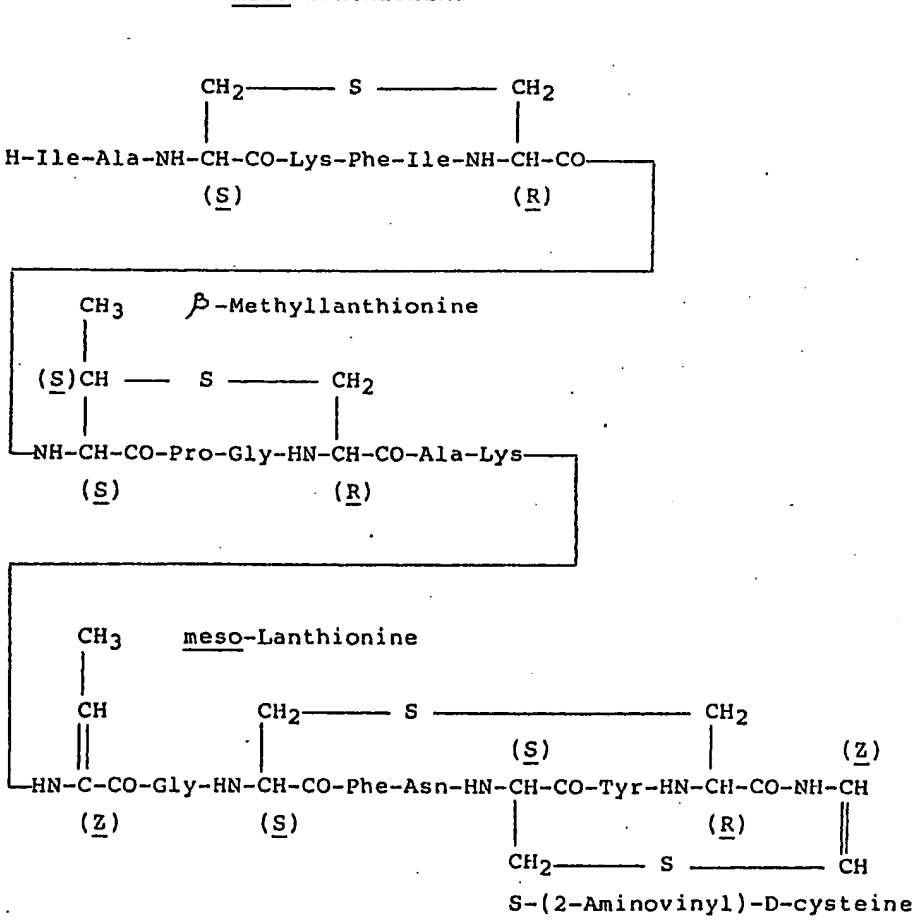
Temperature programme 3 minutes at 80°C, isothermic, then 4°C per minute up to 200°C; carrier gas H_2.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An antibiotically-active polypeptide possessing the following amino acid composition:

Asn (1), Pro (1), Gly (2), Ala (2), Ile (2), Phe (2), Lys (2), Lan (2), β-Me-Lan (1), Dhb (1), Tyr (1), S-(2-aminovinyl)-D-cysteine (1)

and having the following primary structure:



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wherein the halves of the individual thioether amino acids closer to the N-terminus are of D configuration (which corresponds to the S configuration in the R,S nomenclature).

2. An antibiotically-active polypeptide possessing the following amino acid composition:

Asn (1), Pro (1), Gly (2), Ala (2), Ile (2), Phe (2), Lys (2), Lan (2), β-Me-Lan (1), Dhb (1), Tyr (1), S-(2-aminovinyl)-D-cysteine (1) and possessing the following additional parameters:

i) Nature: colourless powder.

ii) Solubility: very readily soluble in mixtures of water/-glacial acetic acid or methanol/glacial acetic acid, soluble in lower alcohols, insoluble in chloroform, acetone, diethyl ether, petroleum ether.

iii) Colour reaction on silica gel plates: ninhydrin, chlorine/TDM, orcin/sulphuric acid, anisaldehyde/sulphuric acid. Non-destructive detection in UV light at 254 nm and by spraying with water.

iv) Thin layer chromatography on ready-made silica gel plates 60 F_{254}:

System A: chloroform/methanol/ 17% ammonia (2/2/1) R_F = 0.73;

System B: chloroform/methanol/ 17% ammonia (70/35/10) R_F = 0.30;

System C: n-butanol/glacial acetic acid/ water (4/1/1) R_F = 0.05.

v) Stability: stable from pH 2 to pH 7; a sharp drop in activity occurs at higher pH values.

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vi) Molecular mass: in the region of 2160 (excluding anions).

vii) Ultra-violet absorption spectrum: in aqueous solution, long-wave maximum at 267 nm.

viii) Infra-red absorption spectrum: strong peaks at about 1200, 1500, 1650 and 3300 cm^{-1}; middle-sized peaks at about 1100 and 1320 cm^{-1} and a double peak at about 2910 to 2950 cm^{-1}.

3. A process for preparing an antibiotically-active polypeptide as defined in claim 1, which comprises culturing Staphylococcus epidermis DSM 3095 aerobically at 34-37°C in a complex nutrient solution consisting of 2 to 4% of a nitrogen source, 1 to 3% of a carbon source, and 0.25 to 1% of a carbonate and/or 0.25 to 0.5% of a hydroxide of an alkaline earth metal.

4. A process as claimed in claim 3, wherein the nitrogen source comprises meat extract.

5. A process as claimed in claim 3, wherein the carbon source comprises a sugar or a sugar alcohol.

6. A process as claimed in claim 5, wherein the carbon source comprises malt extract, maltose, galactose, lactose, mannitol, glucose or glycerol.

7. A process as claimed in claim 3, 4 or 5 wherein the carbonate or hydroxide of an alkaline earth metal used is calcium carbonate or calcium hydroxide respectively.

8. A process as claimed in claim 3, 4 or 5 wherein the complex nutrient solution used consists of 3% meat extract, 2%

malt extract and 0.37% calcium hydroxide.

9. A process as claimed in claim 3 wherein, if casamino acid is used as the nitrogen source, tryptophan and one or more vitamins are also added to the culture medium.

10. A process as claimed in claim 9 wherein the vitamin used is selected from biotin, nicotinic acid, thiamine, pyridoxine, HCl and calcium pantothenate.

11. A process as claimed in claim 3, 4 or 5 wherein the pH of the culture medium is adjusted to a value of between 6.0 and 7.0 prior to fermentation.

12. A process as claimed in claim 3, 4 or 5 wherein the course of production is monitored by continuous sampling.

13. A process as claimed in claim 3 wherein the antibiotic polypeptide thus prepared is subsequently isolated by removing extraneous cells and inorganic salts, concentrating the active substance by n-butanol extraction of the culture liquor at the natural end-pH thereof, evaporating the n-butanol extract, dissolving the residue in methanol, and subsequently freeing the solution from lipid contaminants by ether precipitation.

14. A process as claimed in claim 13 wherein the n-butanol extraction is performed at a pH of 8.0.

15. A process as claimed in claim 3 wherein the antibiotic polypeptide thus prepared is subsequently isolated by adsorbing the culture medium on a polymeric resin based on an acrylate ester or polystyrene, releasing the active substance from the resin by

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elution with a strongly acidic eluant, neutralising the eluate with ammonia, and subsequently concentrating the resulting solution by evaporation in vacuo.

16. A process as claimed in claim 15 wherein extraneous cells and inorganic salts are removed from the culture medium prior to adsorption on the resin.

17. A process as claimed in claim 15 wherein the strongly acidic eluant comprises methanol/concentrated hydrochloric acid in a ratio of 99:1.

18. A process as claimed in claim 3 wherein the antibiotic polypeptide thus prepared and isolated is subsequently purified by subjecting the isolated material firstly to gel chromatography, then to multiplicative counter-current distribution (wherein, in a first, liquid-liquid, distribution system, the active substance is left at the starting point, and subsequently, in a second neutral, distribution system, the active substance moves to a position in the apparatus from which it can conveniently be isolated), and finally to freeze-drying.

19. A process as claimed in claim 18 wherein the gel chromatography is carried out on a dextran gel eluting with methanol/acetic acid in a ratio of 95:5.

20. A process as claimed in claim 18 wherein the liquid-liquid multiplicative counter-current distribution system used is n-butanol/ethyl acetate/0.1 N acetic acid in a ratio of 3:1:3.

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21. A process as claimed in claim 18, wherein the neutral multiplicative counter-current distribution system used is 2-butanol/0.05 N ammonium acetate in a ratio of 1:1.

22. A process as claimed in claims 3 or 4 wherein the polypeptide antibiotic is as defined in claim 2.

23. Staphylococcus epidermidis DSM 3095.

24. Staphylococcus epidermidis DSM 3095 possessing the following characteristics:

Gram coloration	:	positive
Cell size	:	0.5-0.8 µm in diameter
Colony size	:	about 1 mm in diameter
Appearance of colonies	:	smooth, glossy, slightly raised in the centre
Colour of colonies	:	greyish-white
Cells in culture	:	often single cells or groups of two, seldom larger clumps
Haemolysis	:	cultures spread on blood slides showed strong haemolysis
Anaerobic growth	:	the strain grows even

		under anaerobic conditions
Lysozyme sensitivity	:	the cells are resistant to lysozyme in quantities of up to 2 mg/ml
Lysostaphin sensitivity	:	the cells are sensitive to lysostaphin even in quantities of 20 µg/ml
Epidermin resistance	:	demonstrated up to 1 mg/ml
Other resistances	:	in liquid culture the strain shows a marked resistance to Streptomycin (up to 1 mg/ml) and Spectinomycin (0.5 mg/ml)
Increased NaCl content	:	the strain still grows well with a sodium chloride content of up to 15% by weight,

the colonies or the spread-out cultures being adhesive.

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25. A strain of Staphylococcus epidermidis according to claim 24 capable of producing an antibiotic polypeptide as defined in claim 1 or 2, and being itself resistant thereto.

26. A process for preparing an antibiotically-active polypeptide possessing the following amino acid composition:

Asn (1), Pro (1), Gly (2), Ala (2), Ile (2), Phe (2), Lys (2), Lan (2), -Me-Lan (1), Dhb (1), Tyr (1), S-(2-aminovinyl)-D-cysteine (1) and having the following primary structure:

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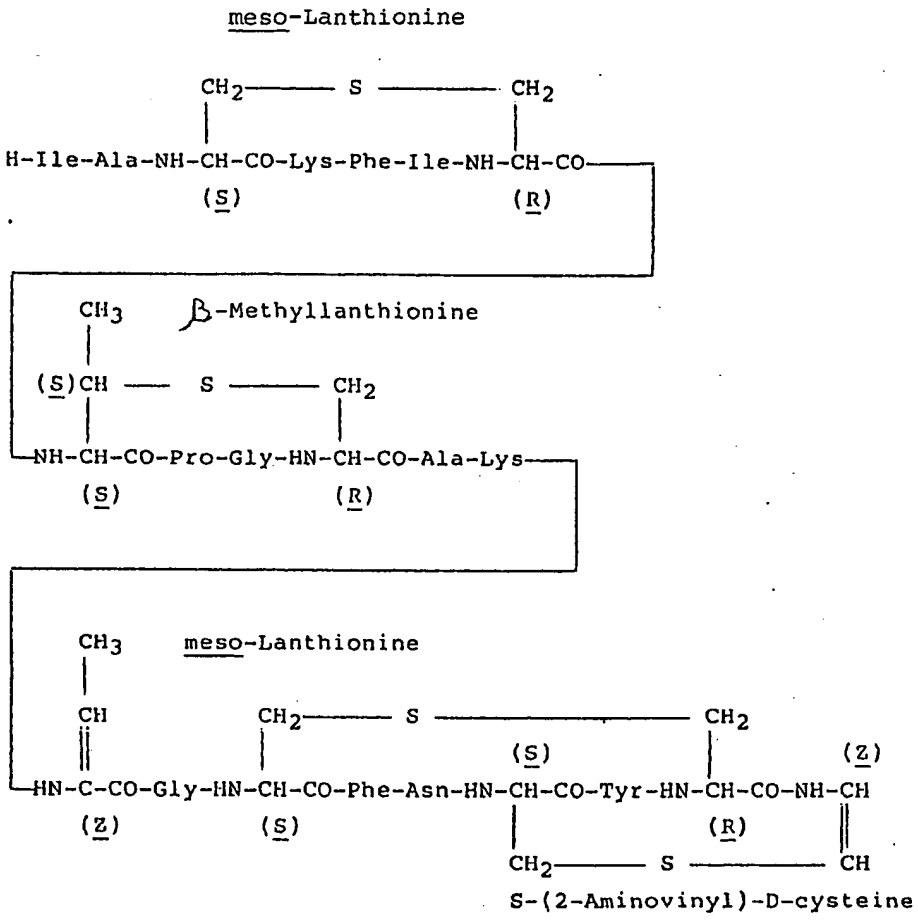

wherein the halves of the individual thioether amino acids closer to the N-terminus are of D configuration (which corresponds to the S configuration in the R,S nomenclature), which comprises culturing Staphylococcus epidermidis DSM 3095.

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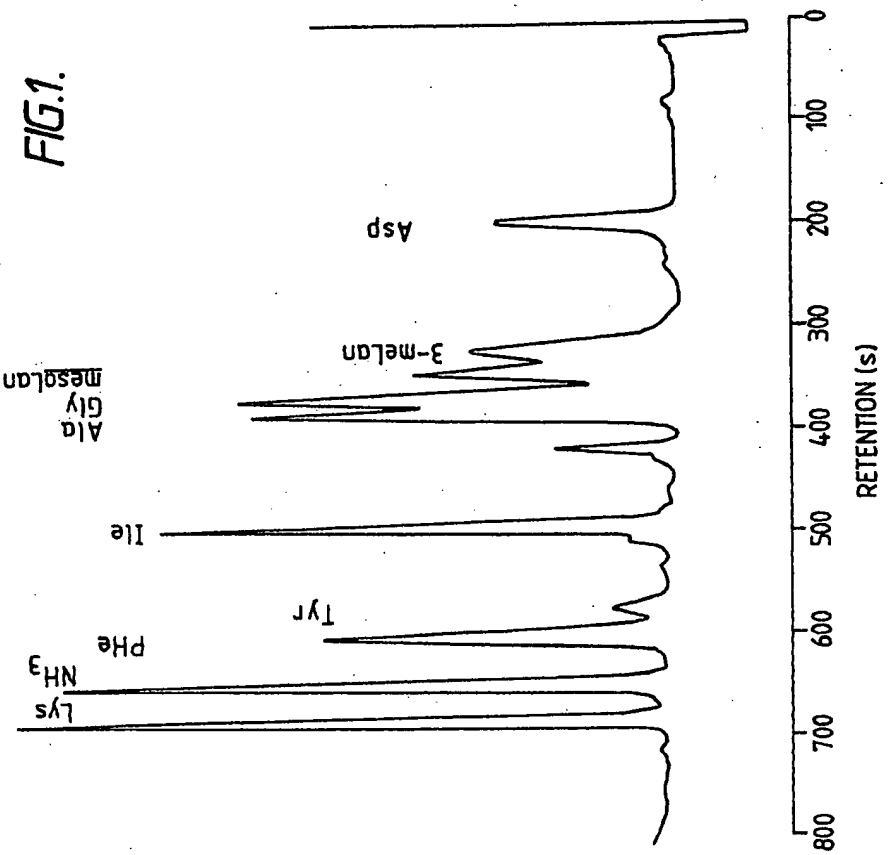
Abstract

The specification describes a polypeptide, referred to as epidermin, having an antibiotic activity, a process for preparing it, a new strain of Staphylococcus epidermidis DSM 3095 resistant to this substance, antibiotic preparations containing the active substance epidermin and the use of epidermin for fighting infectious diseases.

FIG.1.



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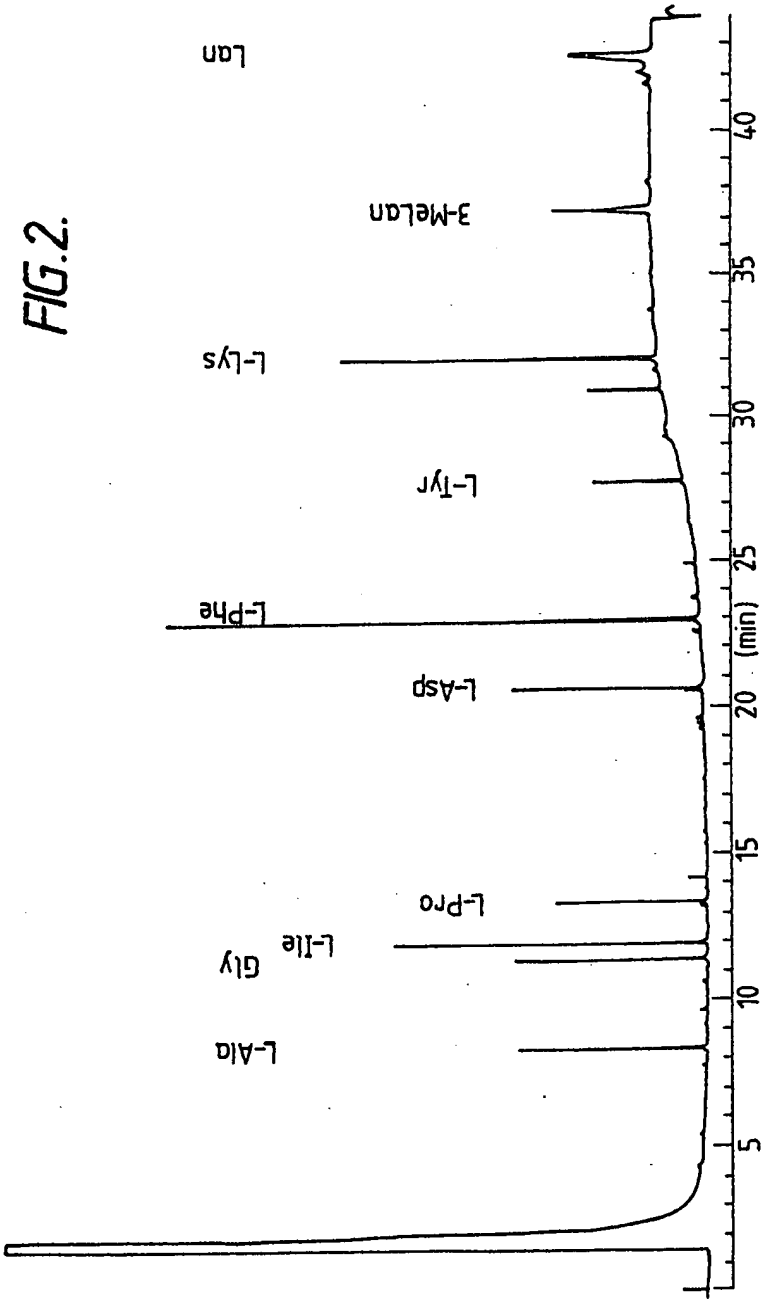

FIG.2.

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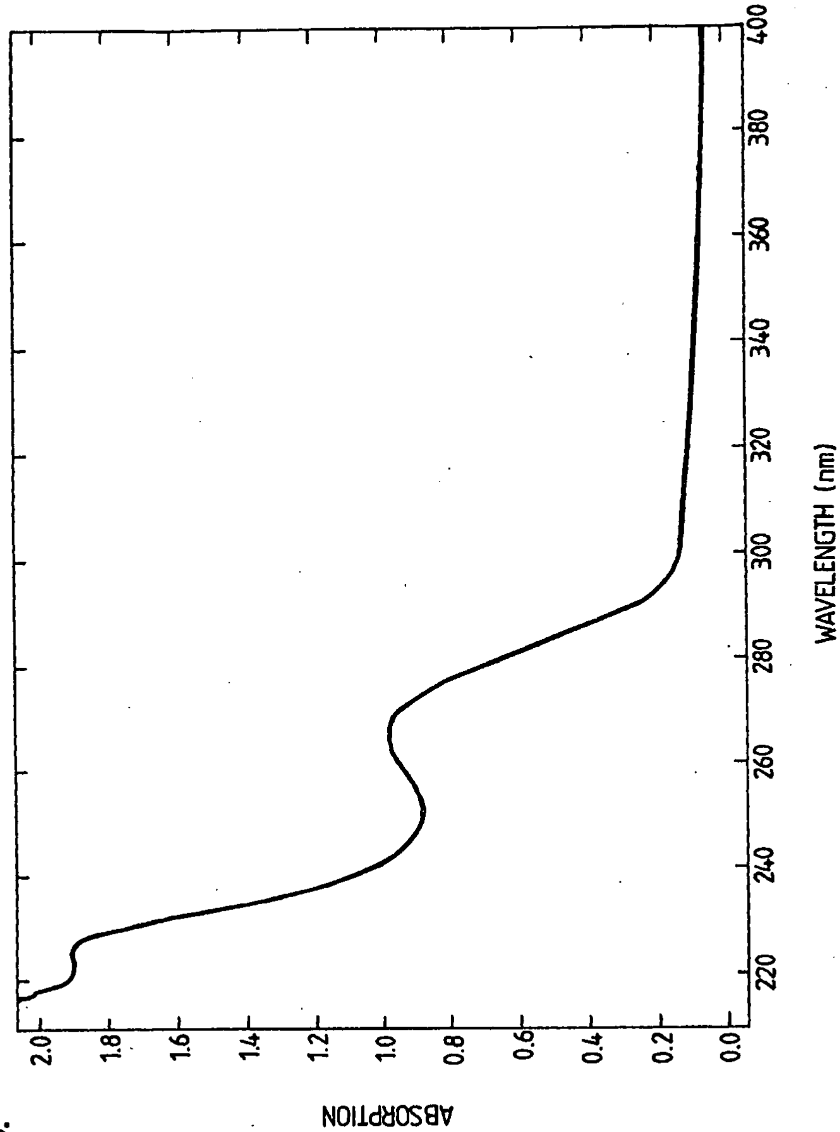

FIG.3.

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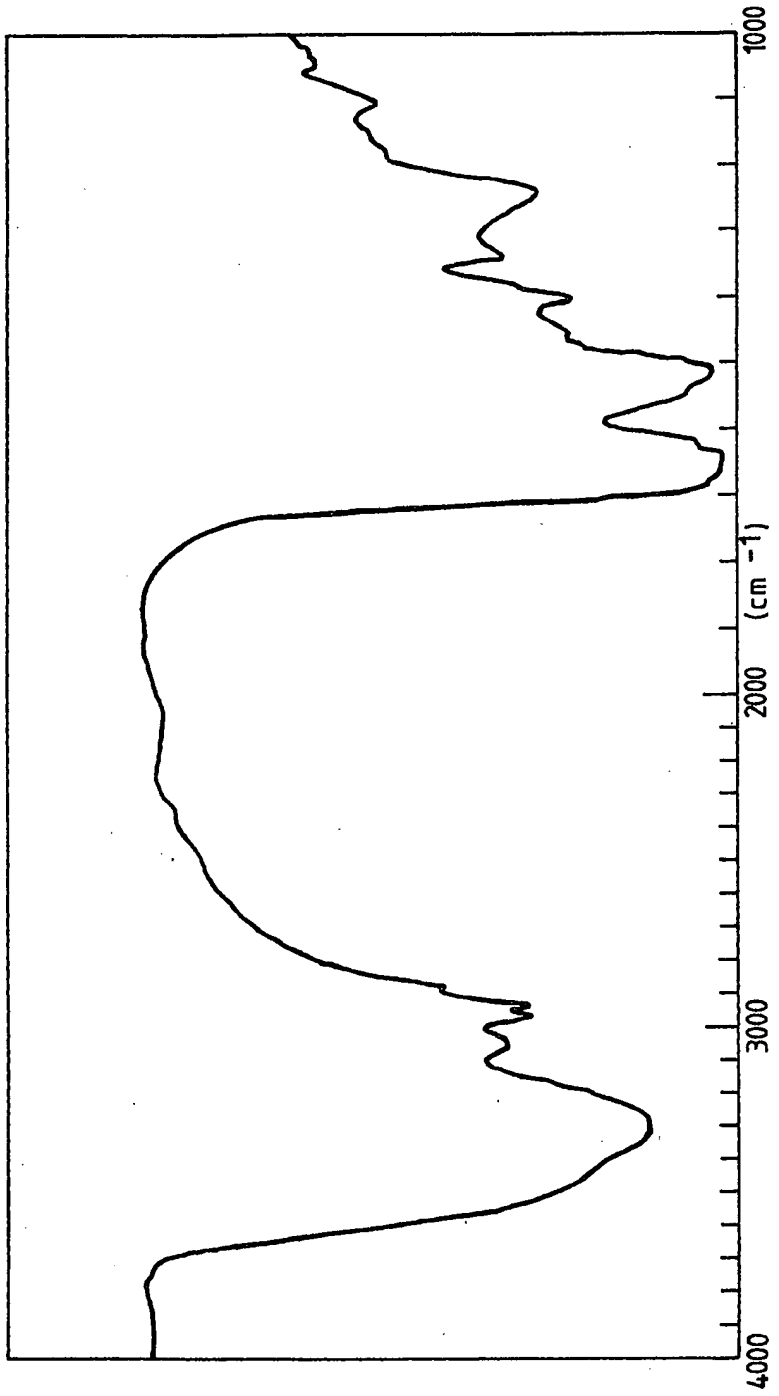

FIG. 4.

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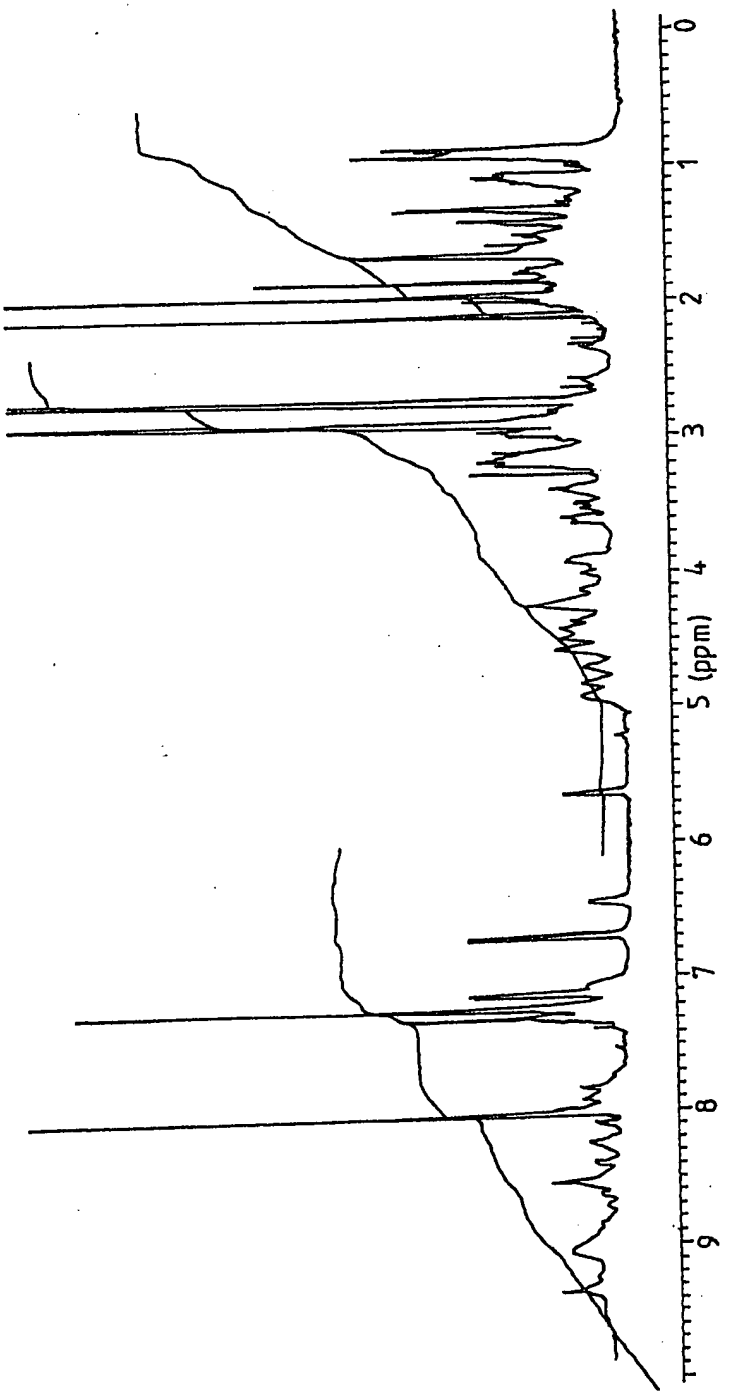
FIG.5.



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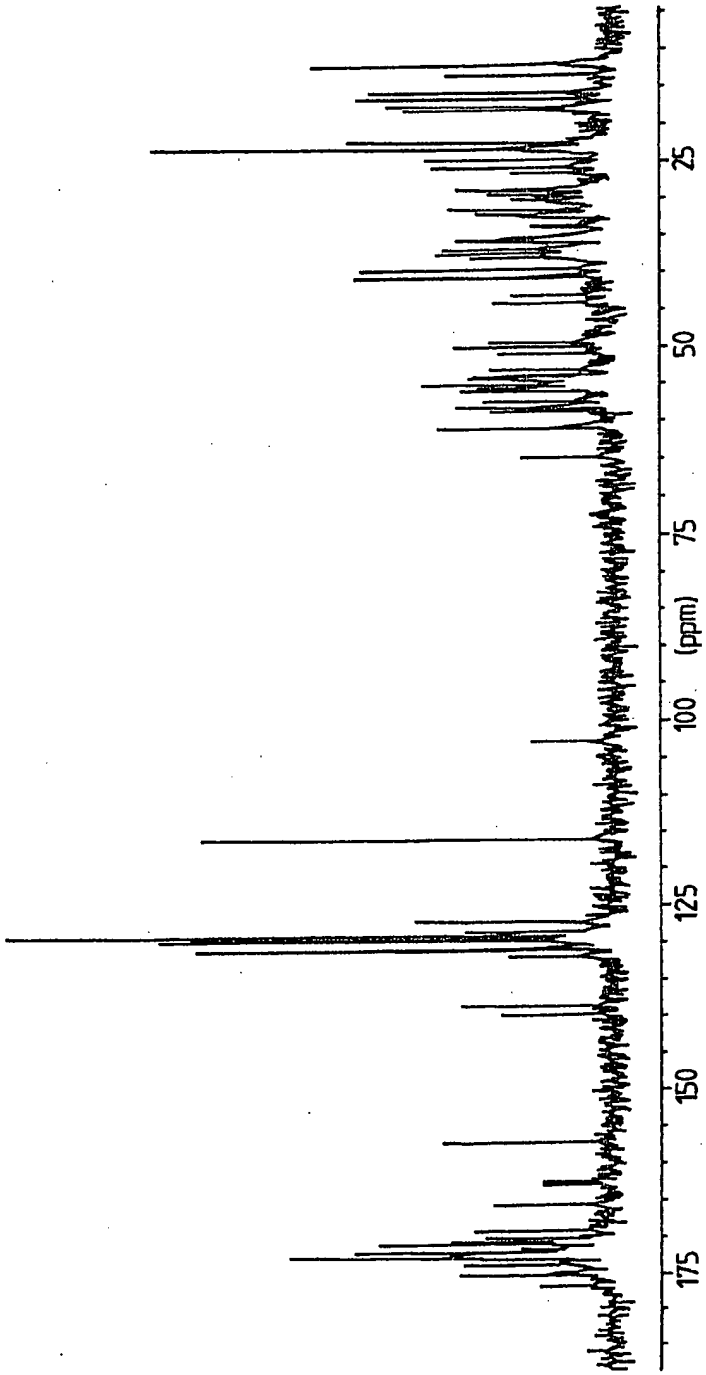

FIG.6.

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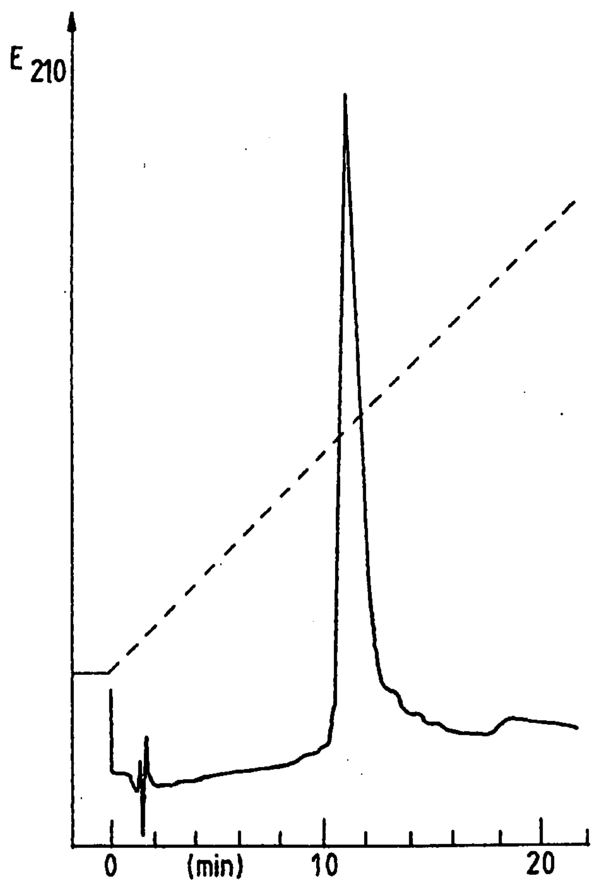
FIG.7.



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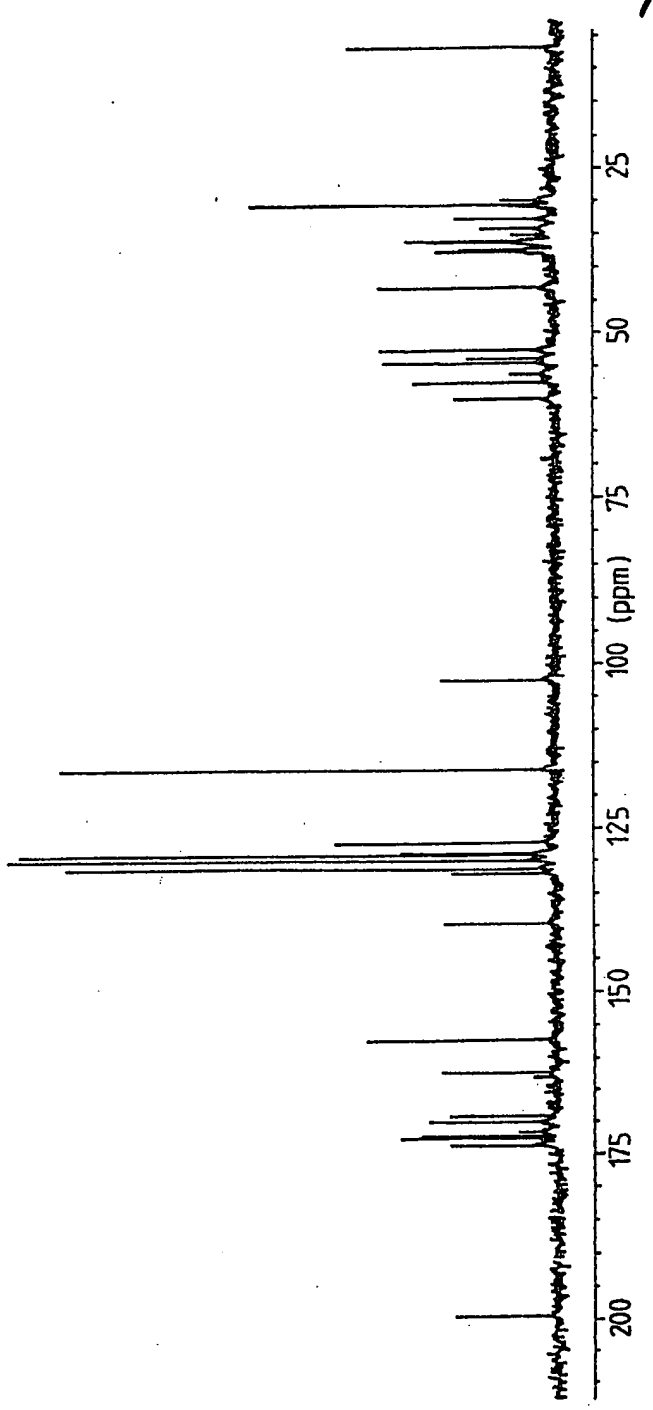

FIG. 8.

FIG.9.

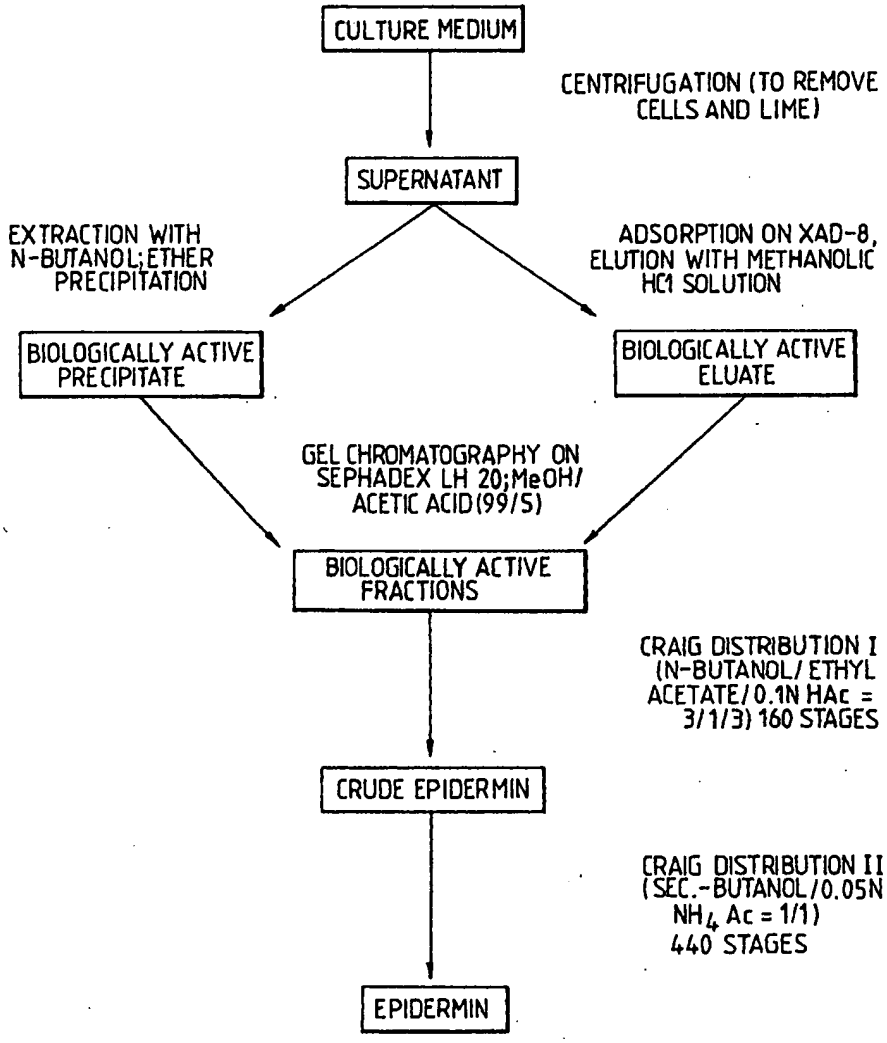

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FIG.10.

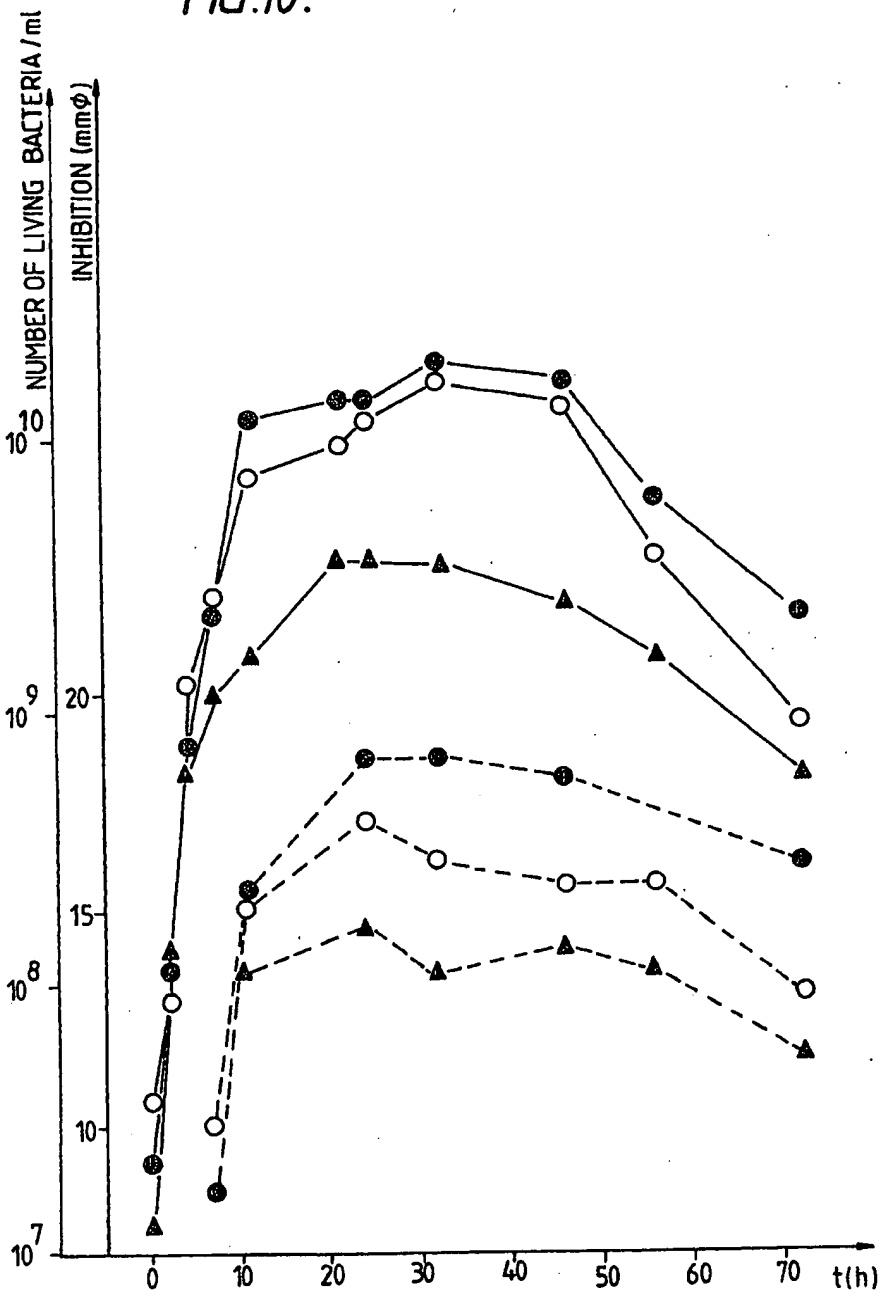

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FIG.11.

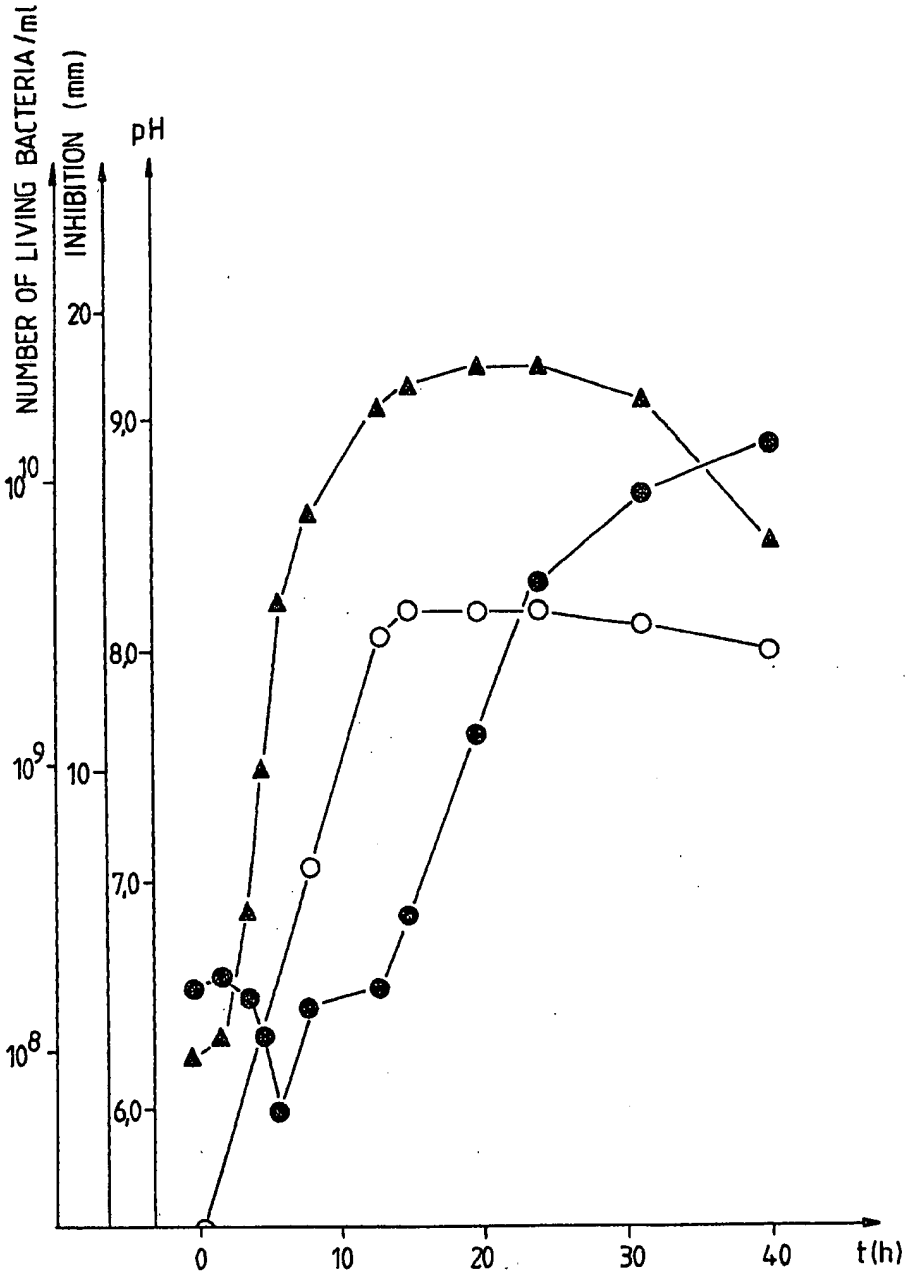

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FIG.12.

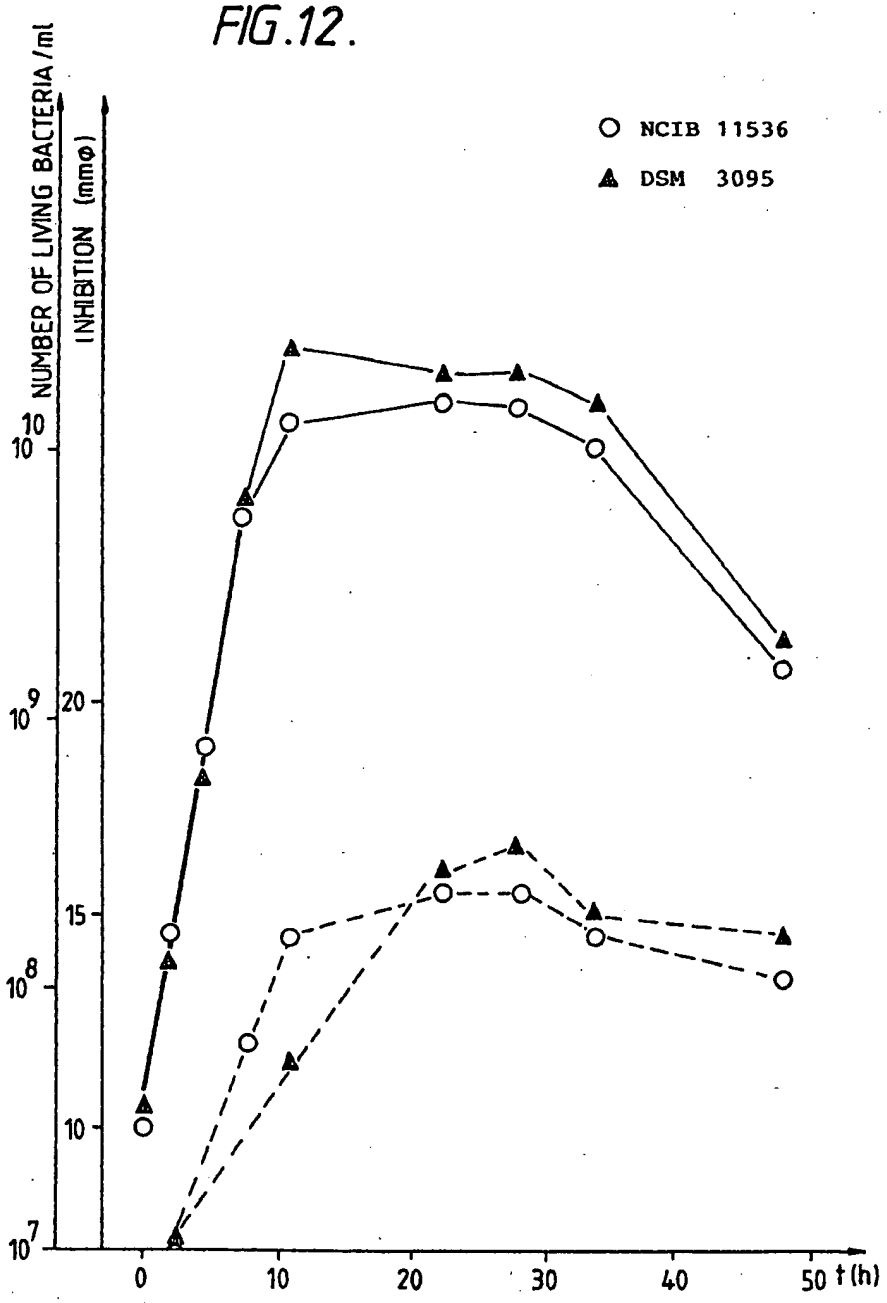

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FIG.13.

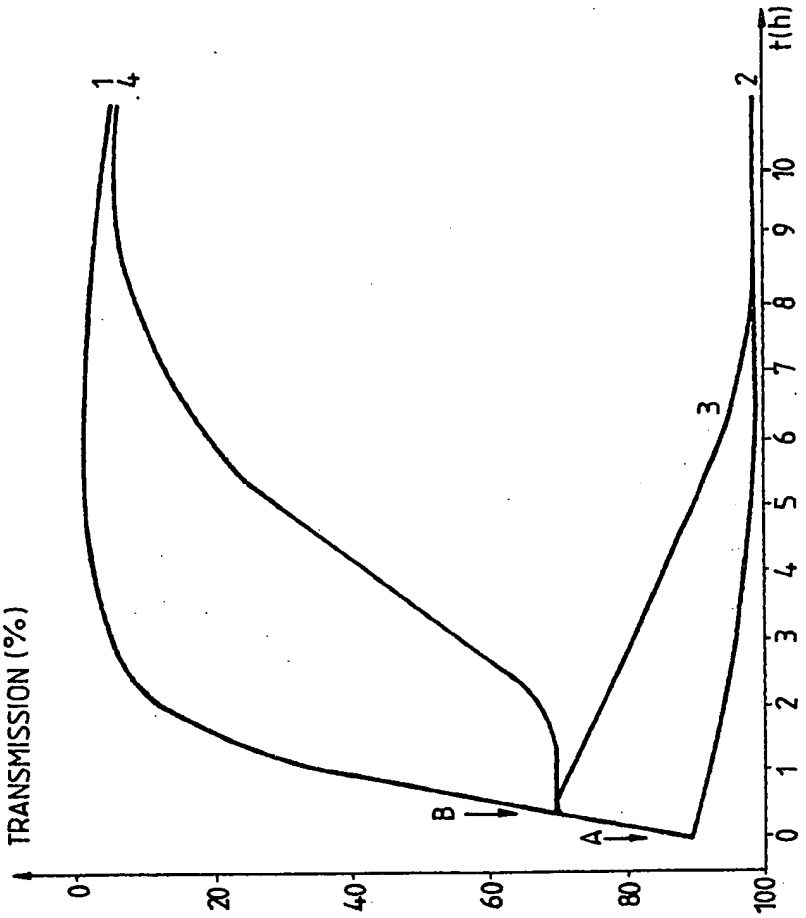

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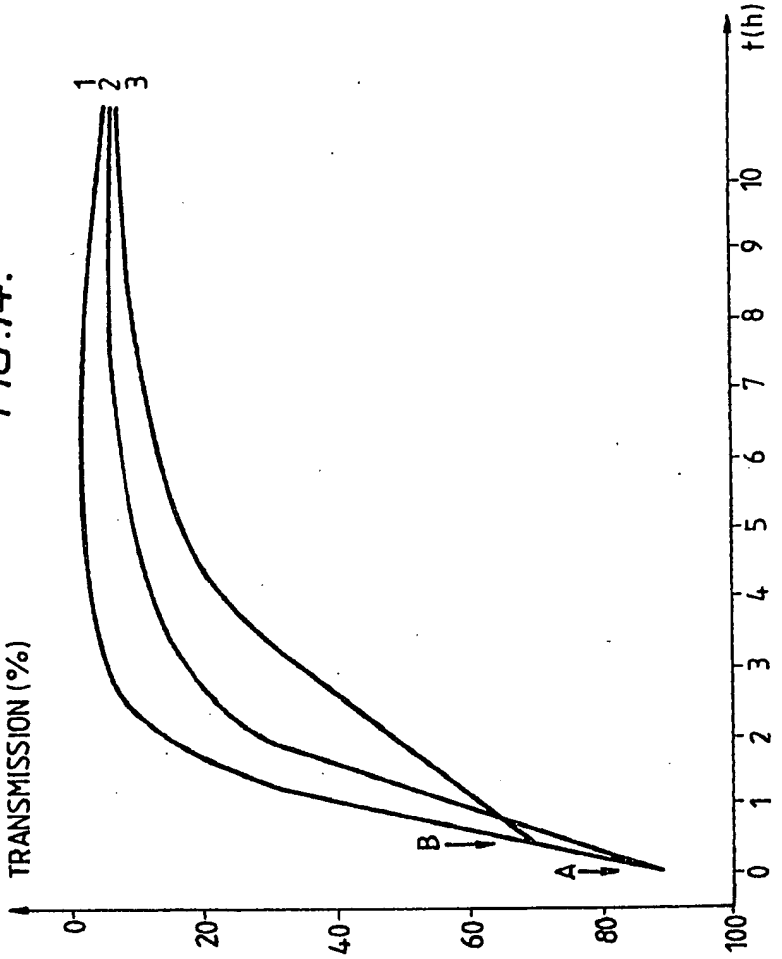
FIG.14.



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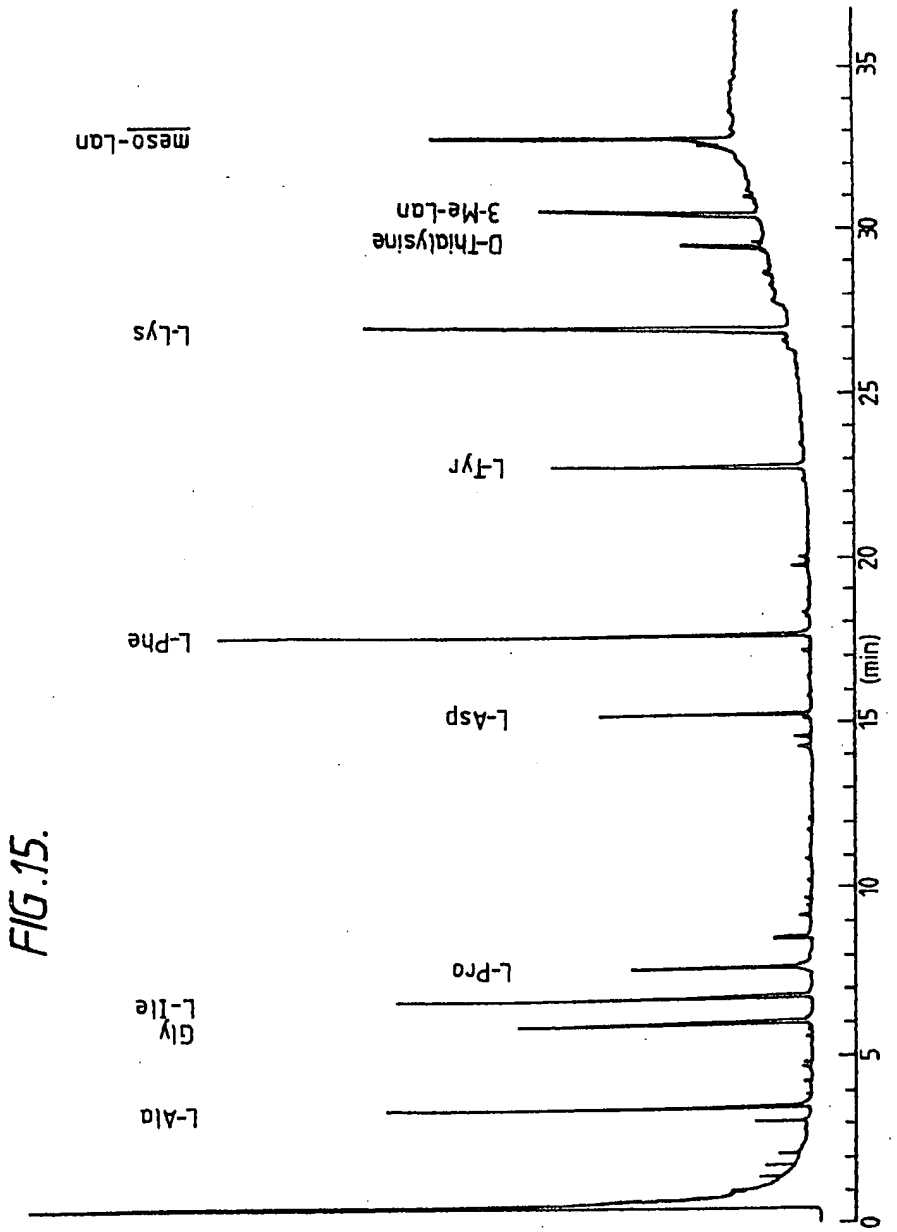

FIG.15.

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